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Toxicity Resistance in Mummichog (Fundulus heteroclitus) from a Chemically Contaminated Environment

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College of William and Mary - Virginia Institute of Marine Science

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TOXICITY RESISTANCE IN MUMMICHOG (*FUNDULUS HETEROCLITUS*) FROM A CHEMICALLY CONTAMINATED ENVIRONMENT

A Thesis

Presented to

The Faculty of the School of Marine Science

The College of William and Mary in Virginia

In Partial Fulfillment

Of the Requirements for the Degree of

Master of Art

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by

Cynthia A. H. Williams

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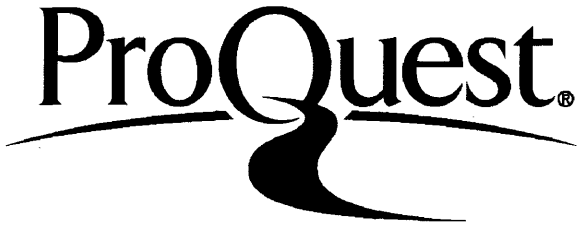

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This thesis is submitted in partial fulfillment of

the requirements for the degree of

Master of Arts

Cynthia A. H. Williams

Cynthia A. H. Williams

Approved, October 1994

Wolfgang K. Vogelbein, Ph.D.
Committee Co-Chairman/Advisor

Robert J. Huggett, Ph.D.
Committee Co-Chairman/Advisor

John E. Graves, Ph.D.

Morris H. Roberts, Jr., Ph.D.

Michael A. Unger, Ph.D.

Peter A. Van Veld, Ph.D.

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ABSTRACT

A growing field in toxicity research focuses on acute and chronic health problems in aquatic organisms living in chemically contaminated environments. Fishes exposed to environmental toxicants may suffer adverse effects ranging in severity from relatively minor sublethal changes to death. Certain populations, however, have been shown to develop resistance to these toxicants, either by physiological acclimation or by genetic adaptation.

The highly industrialized Elizabeth River, Virginia, is one of the most polluted waterways in the United States. Studies at the Virginia Institute of Marine Science (VIMS) have focused on a population of mummichog (*Fundulus heteroclitus*) from a contaminated site in the southern branch of this river, which has been shown to contain high sediment concentrations of polycyclic aromatic hydrocarbons, presumably of creosote origin. While these fish exhibit a high prevalence of neoplastic and non-neoplastic tissue lesions, their embryos apparently are resistant to the acute toxicity of the pollutants found at the site; a resistance which does not appear to be shared by mummichog embryos from a population living in a relatively uncontaminated environment. The purpose of this study was to characterize the resistance occurring in these fish embryos.

Short-term sediment exposure tests using mummichog embryos from the contaminated population and from a reference population were conducted to evaluate the differential resistance exhibited by these embryos, to determine if the observed resistance appeared to be due to physiological acclimation or to genetic adaptation, and to document the effects of contaminated sediment on non-resistant embryos. In addition, exposure tests using hybrid embryos from controlled crosses between the two populations were conducted to investigate the nature of the inheritance of the resistance trait. Embryo responses to the various treatments used in these experiments were assayed using mortality and cardiovascular malformation as toxicity end points.

In all experiments, Elizabeth River embryos exposed to contaminated sediment developed and hatched relatively normally, while similarly exposed reference embryos developed cardiovascular abnormalities and died. The cardiac terata most often observed in these experiments were

tube hearts of varying severity, accompanied by pericardial edema. Hybrid embryos, for the most part, responded to contaminated sediment in a manner similar to reference embryos. However, hybrids obtained by crossing Elizabeth River females with reference males appeared to be slightly less susceptible to the toxic effects of contaminated sediment than were the reciprocal hybrids.

The results of this study supported the hypothesis that mummichog embryos from a chemically contaminated site in the Elizabeth River, Virginia, are more resistant to the acute toxicity of contaminated sediment than are embryos from an uncontaminated reference site. This resistance was apparent when either mortality or cardiovascular malformation was used as a criterion for toxic effects. The observed resistance was shown to be due to genetic adaptation rather than physiological acclimation. It appears to be inherited as an autosomal recessive trait which may be modified by maternal effects.

INTRODUCTION

Toxicity Resistance in Aquatic Organisms: Acclimation versus Adaptation

Aquatic organisms inhabiting chemically contaminated environments may develop resistance to local pollutants over time. Resistance, or tolerance, can be defined as the ability of individual organisms to cope with exposure to toxicant concentrations that are inhibitory or lethal to nontolerant individuals (Mulvey and Diamond 1991). Tolerance may be acquired by physiological acclimation or genetic adaptation (Angus 1983; Klerks and Weis 1987; Weis and Weis 1989a; Mulvey and Diamond 1991). Physiological acclimation can result from low-level, nonlethal exposure. This type of resistance is lost when fish are moved to clean water and is not passed on to offspring (Klerks and Weis 1987; Weis and Weis 1989a; Mulvey and Diamond 1991). Genetic resistance can develop when chronic or recurrent exposure to toxic concentrations of a contaminant selects individuals with toxicant resistant genotypes. This type of resistance is not lost when fish are moved to clean water and is passed on, even to offspring reared in an unpolluted environment (Angus 1983; Andreasen 1985; Klerks and Weis 1987; Weis and Weis 1989a; Mulvey and Diamond 1991).

Physiological acclimation has been demonstrated in several studies of fishes exposed to environmental pollutants, particularly to metals. Bensen and Birge (1985), for example, found that fathead minnows (*Pimephales promelas*) from a metal-contaminated flyash pond were significantly more tolerant of cadmium and copper than were hatchery minnows. Furthermore, when ash pond minnows were held in clean water for a week or more, their tolerance decreased significantly, while tolerance in hatchery minnows was increased following acclimation to sublethal concentrations of cadmium. Bensen and Birge (1985) attributed the observed tolerance induction, at least in part, to the increased production of metallothioneins, proteins that selectively bind and deactivate metals. This assertion was based on biochemical studies which demonstrated that gill metallothionein concentrations correlated closely with the fluctuations in tolerance seen during cadmium acclimation and deacclimation.

Dixon and Sprague (1981a,b) investigated metal acclimation in rainbow trout (*Oncorhynchus mykiss*) and found that fish pre-exposed to sublethal levels of arsenic or copper developed increased tolerance of these metals. Rainbow trout pretreated with low levels of aluminum showed increased resistance to the toxic effects of this metal as well (Orr et al. 1986). Dixon and Sprague (1981c) attributed copper acclimation in rainbow trout to the

increased synthesis of a low molecular weight hepatoprotein. Since this protein was not purified and characterized, it could not be specifically identified as metallothionein; however, the methods used to isolate the hepatoprotein fraction would have ensured the inclusion of metallothionein.

Physiological acclimation also has been reported in fishes exposed to organic pollutants. Norup (1972) found that guppies (*Poecilia reticulata*) pre-exposed to a low level of pentachlorophenol (PCP) survived longer when exposed to higher levels of PCP than did guppies that were not pre-exposed. Angus (1983) investigated phenol tolerance in mosquitofish (*Gambusia affinis*) from polluted and nonpolluted sites, and found evidence for both physiological acclimation and genetic adaptation. Acclimation was indicated by the results of laboratory exposure experiments, in which some phenol-resistant fish lost their resistance after being held in clean water, while some phenol-susceptible fish developed resistance after repeated nonlethal exposures. Based on these results and the rapidity of the toxic response (phenol-sensitive fish showed signs of intoxication within 15 minutes of exposure), Angus (1983) hypothesized that phenol tolerance was due to the induction of a rapidly acting detoxication mechanism, most likely an enzyme or an enzyme system. Similarly, Weis and Weis (1989a) suggested that pre-exposure to organic

pollutants may cause increased tolerance by activation of a microsomal mixed-function oxidase system (MFO), which converts organic toxicants into excretable metabolites. In studies of insecticide resistance in mosquitofish, Fabacher and Chambers (1973) found that resistant fish exhibited increased levels of MFO enzymes, most likely due to a combination of environmental induction and genetic inheritance. However, as this did not completely explain the observed pyrethroid tolerance, they postulated the involvement of another resistance mechanism as well, such as desensitization of nervous tissue.

Increased tolerance of toxicants cannot always be attributed to physiological acclimation alone. In many cases, tolerance involves a change in the genetic structure of a fish population; i.e., pollution acts as a source of "unnatural selection" for tolerant fish (Angus 1983). In one of the earliest studies of this phenomenon, mosquitofish from waters contaminated with insecticides were found to be significantly more resistant to DDT than were fish from uncontaminated waters (Vinson et al. 1963). These fish also were found to be highly resistant to most organochlorine insecticides (Culley and Ferguson 1969), as well as to pyrethroids (Fabacher and Chambers 1973). Genetic adaptation was assumed to be at least partially responsible for the observed pyrethroid resistance, as the progeny of resistant fish kept in clean water maintained some degree of

tolerance. In further studies of mosquitofish resistance to insecticides, Andreasen (1985) found that the laboratory-reared F_1 progeny of tolerant fish maintained a pronounced resistance to toxaphene. This suggested that resistance was due to natural selection of a genetic trait present in the fish population.

Genetic adaptation also has been observed in fish exposed to pollutants other than pesticides. As explained earlier, Angus (1983) found that both physiological acclimation and genetic adaptation appear to be involved in phenol tolerance in mosquitofish. Selective pressure was indicated by the significantly higher proportion of phenol-resistant fish in a contaminated stream than in a relatively clean lake. Based on these results, Angus postulated that the capacity to induce phenol resistance (via a detoxifying enzyme system) is genetically determined. Weis et al. (1981a) investigated methylmercury tolerance in mummichog (*Fundulus heteroclitus*) and found that embryos from a contaminated estuary were much more tolerant of meHg than were embryos from a relatively unpolluted habitat. This resistance appeared to have a genetic basis, because when females from the polluted site were kept in clean water, they continued to produce tolerant eggs. In addition, tolerance was correlated with the fin ray count of the female, a characteristic having a large genetic component (Weis et al. 1982).

Inheritance of Toxicity Resistance

Genetically based toxicant resistance may be controlled by one or a few genes with major effects (Mendelian inheritance) or by many genes with minor effects (non-Mendelian or polygenic inheritance; Kleeberger and Levitt 1991; Mulvey and Diamond 1991). The key difference between Mendelian traits and quantitative, or polygenic, traits is the amount of phenotypic variation between genotypes as compared with the amount of variation within genotypes (Suzuki et al. 1986). For quantitative traits, the phenotypic differences between genotypic classes are small as compared with individual variation, which usually leads to a continuous distribution of phenotypes. For Mendelian traits, the phenotypic differences between genotypes tend to be larger, leading to discrete phenotypic classes (Suzuki et al. 1986; Mulvey and Diamond 1991).

The mode of inheritance of a resistance trait can be determined using breeding studies in which F_1, F_2 and back-cross progeny from tolerant and nontolerant groups of organisms are tested for toxicity resistance (Festing and Blackwell 1988; Kleeberger and Levitt 1991; Mulvey and Diamond 1991). The observation of acceptable Mendelian ratios in the progeny of these crosses suggests (but does not prove) a single gene mode of inheritance, whereas failure to observe Mendelian ratios is suggestive of

polygenic inheritance (Festing and Blackwell 1988). It should be noted, however, that genetic analysis can be complicated by factors such as maternal effects, egg cytoplasmic effects, epistasis and variable penetrance or expressivity (Suzuki et al. 1986; Tave et al. 1989). In these cases, the key is to understand that the most important features of Mendelian inheritance are not specific ratios, but rather the predictability of the types of offspring that will result from certain crosses.

Most experimental animal studies have suggested that xenobiotic response is under polygenic control (Kleeberger and Levitt 1991). However, xenobiotic resistance is a complex phenomenon; one which could result from several different adaptive mechanisms that are likely to vary depending on the population, species and specific contaminants involved (Klerks and Weis 1987; Mulvey and Diamond 1991). Unfortunately, there have been very few studies on the inheritance of toxicity resistance in fishes, so it is too early to discern any general trends. Yarbrough et al. (1986) investigated cyclodiene insecticide resistance in mosquitofish, and found it to be inherited as a single, autosomal, intermediate gene. Toppin et al. (1987) analyzed mercury resistance in mummichog embryos, and while they did not determine the specific mode of inheritance of the resistance trait, they did discover that it is maternally controlled. As can be seen by the results of just these two

studies, the genetics of toxicity resistance in fish is a complex issue which needs further research.

The literature on the genetics of fish resistance to diseases and environmental stresses is older and larger than that on the genetics of toxicity resistance, and thus provides useful background for future toxicity studies. Some of the published research includes studies on fish resistance to viral diseases (Amend and Nelson 1977; Kaastrup et al. 1991), to fungal infection (Hanke et al. 1991), to parasitic infection (Ibarra et al. 1992), and to temperature extremes (Tave et al. 1989; Fujio et al. 1990; Kanda et al. 1992). Chevassus and Dorson (1990) provided a good review of aspects of the genetics of resistance to disease in fishes. Among their many conclusions, they found that in most F1 crosses between resistant and susceptible populations, disease susceptibility appears to be recessive. However, they pointed out that studies of F2 crosses and backcrosses need to be done before the genetics of these resistance traits can be understood.

Use of Embryos in Aquatic Toxicity Research

Embryos and larvae are usually the most sensitive stages in the life history of fishes (Weis and Weis 1989b; 1991; Sharp 1991). Toxicologists have used this knowledge

to develop early life stage tests to replace whole life cycle toxicity tests for the assessment of overall toxicity to a species. In addition to their sensitivity, fish embryos exhibit other characteristics that can be useful for toxicity testing: large numbers of embryos can be obtained from most fishes, enabling better replication and data analysis; the developmental time of many species is relatively short; and the transparency of the chorion allows for continuous and detailed examination of the embryo throughout the test (Weis and Weis 1989b; 1991).

In recent years, numerous studies have been performed on the effects of various toxicants on the embryos and larvae of marine and freshwater fish species (Sharp 1991). The usual parameters analyzed in these studies include hatch, survival and growth rates, although in some cases, the development of terata is noted as well (Weis and Weis 1989b). An excellent review of research in this field is provided by Weis and Weis (1989b), who also summarize in tabular form the various toxicants and fish species used and the effects observed.

While most toxicologists use percent viable hatch as an expedient endpoint, much more toxicity information can be obtained by screening embryos for the development of terata. Teratogens tend to be nonspecific in terms of the types of defects they cause; the timing of exposure appears to be more important than the type of chemical involved (Weis and

Weis 1989b; 1991; Sharp 1991). Fish embryos tend to develop certain types of abnormalities. The skeletal system appears to be the most sensitive, with scoliosis, lordosis and stunting frequently observed in many species exposed to a variety of toxicants. The circulatory system is another common site for abnormalities, including circulatory stasis, lack of bending of the heart tube, and pericardial edema. Finally, embryos also frequently exhibit optical malformations, such as microphthalmia, anophthalmia, cyclopia and lesser degrees of fusion of the optic vesicles (Weis and Weis 1989b; 1991; for pictures of these defects, see Sharp 1991). A classification system which ranks the relative severity of skeletal, cardiovascular and craniofacial defects has been developed to allow quantitation of the teratogenic effects observed in toxicity studies (Weis and Weis 1977b; 1982; Weis et al. 1981a,b).

One group of researchers is using the teratogenic response of fish embryos to study population differences in resistance to environmental contaminants. As mentioned earlier, Weis et al. (1981a) found that *Fundulus heteroclitus* embryos from a polluted environment were much less susceptible to the teratogenic effects of methylmercury than were embryos from a relatively pristine site. The average abnormality index values for batches of embryos from the polluted site generally were low (indicating resistance), while values for batches of control embryos

ranged from low to high. In a subsequent study, also mentioned earlier, Toppin et al. (1987) used the same abnormality indices to assess the resistance of hybrid embryos obtained from crosses of males and females from the two populations. This research demonstrated one advantage of using embryos in toxicity resistance studies; specifically, the amount of time that can be saved in conducting genetic analyses. The resistance of F1 and later generations can be tested immediately, rather than after months of growth and development, as is the case when adult fish are used.

Elizabeth River Research

The Elizabeth River, Virginia, is a highly industrialized tributary of the James River near the mouth of the Chesapeake Bay. Sediments in the river have been shown to be heavily contaminated with polycyclic aromatic hydrocarbons (PAH) at concentrations which are among the highest found anywhere in the United States (Bieri et al. 1986). Concentration maxima occur in the southern branch of the river, particularly in the vicinity of three wood treatment facilities. Bieri et al. (1986) attributed much of this contamination to two massive spills of creosote (either pure or mixed with coal tar) which occurred at one of these facilities during the 1960s. PAH are the major

constituents of coal tar creosote, along with small amounts of phenolic and heterocyclic compounds (Mueller et al. 1989).

Several recent studies indicate that fishes in the Elizabeth River exhibit a variety of adverse effects attributed to exposure to contaminated sediments. Huggett et al. (1987) observed high prevalences of fin erosion in hogchoker (*Trinectes maculatus*) and toadfish (*Opsanus tau*) from the southern branch of the river, as well as high prevalences of cataracts in spot (*Leiostomus xanthurus*), weakfish (*Cynoscion regalis*) and croaker (*Micropogonias undulatus*). Cataract prevalence in these fishes was correlated with pollution load (as estimated by sediment PAH concentrations). Weeks and Warinner (1984) and Weeks et al. (1986) demonstrated that macrophages from Elizabeth River spot and hogchoker have diminished chemotactic and phagocytic activity as compared to macrophages from control fish. Vogelbein et al. (1990) discovered a population of mummichog (*Fundulus heteroclitus*) with a high prevalence of liver cancer at a heavily contaminated site in the southern branch of the river. This site is located adjacent to Atlantic Wood Industries Inc., a wood processing facility which had been active for more than sixty years when operations were suspended in 1991 (Buchman et al. 1992). Sediments at this site were found to contain several toxicants, including extremely high concentrations of PAH,

which were presumed to have originated from the Atlantic Wood facility (Vogelbein et al. 1990). Hepatic lesions were found in 93% of the mummichog examined, and 33% of the fish had hepatocellular carcinoma. In contrast, no hepatic lesions were detected in mummichogs from two less contaminated sites, including one which is located directly across the river from the Atlantic Wood facility.

While conducting preliminary laboratory exposure studies with sediments from the Atlantic Wood site, Vogelbein and Van Veld (unpublished results) made an unexpected observation: Atlantic Wood (AW) mummichog embryos appeared to be more tolerant of the acute toxicity of these sediments than were embryos from a reference site. These preliminary results suggested that AW mummichog had developed some type of resistance to the acute toxicity of the pollutants in their environment.

The mummichog has been used extensively as a test organism in toxicological studies and as an indicator of marine water quality (Eisler 1986). Some of its useful attributes include a wide geographic distribution in shallow bays, inlets and tidal creeks along the Atlantic coast (Bigelow and Schroeder 1953), abundance throughout much of this range, and a limited summer home range of approximately 30-40 meters (Lotrich 1975). In addition, mummichog exhibit hardiness in captivity and adapt well to laboratory conditions (Atz 1986; Eisler 1986). They can be induced to

spawn throughout the year (Boyd and Simmonds 1974), and the eggs can be artificially fertilized and incubated in the laboratory (Armstrong and Child 1965; Atz 1986). The developmental stages of the embryos have been well described (Armstrong and Child 1965), and their hardiness makes them ideal for experimental manipulation.

Objectives

The overall purpose of this research project was to characterize the acute toxicity resistance to contaminated sediments observed in preliminary experiments with mummichog embryos from the AW site.

The specific objectives of this study were to:

1) Evaluate the differential resistance exhibited by embryos from contaminated and reference sites when exposed to various mixtures of contaminated sediments.
2) Describe the effects of contaminated sediments on non-resistant embryos.
3) Determine if the observed resistance is due to physiological acclimation or to genetic adaptation.
4) Document the inheritance of the resistance trait in purebred resistant and susceptible embryos as well as in hybrid embryos.

To address each of these objectives, a series of short-term sediment exposure tests was conducted using mummichog embryos from the contaminated AW population and from a reference population, as well as embryos from lab-maintained fish and hybrid embryos from controlled crosses between the two populations.

MATERIALS AND METHODS

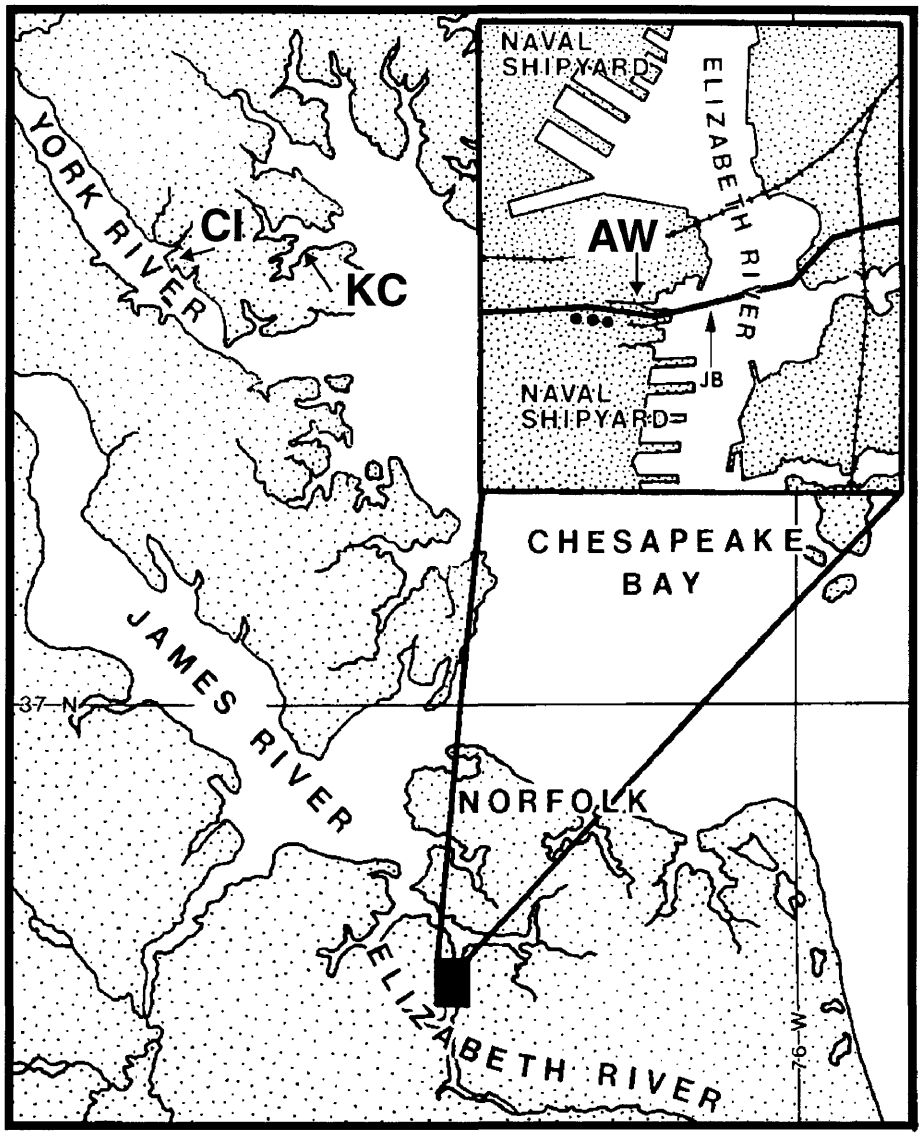
Sample Sites

The research site for this project was a contaminated tidal creek in the Elizabeth River, Virginia, adjacent to Atlantic Wood Industries Inc. (AW), a recently closed wood treatment facility. The reference site was an inlet adjacent to Catlett Island (CI) in the York River, Virginia; an area which has been designated a NOAA National Estuarine Research Reserve System (NERRS) site. In addition, King Creek (KC) in Gloucester Point, Virginia was used as a source of reference fish for certain preliminary studies. The location of each of these sites is marked in Figure 1.

Sediment Collection and Preparation

Surficial sediments (top 5-10 cm) were collected from AW and CI using a Ponar grab sampler and stored in plastic buckets at 10°C until needed. The first batch of sediment was collected in May 1992 and used for a preliminary flow-through exposure study in July 1992. A second batch of sediment was collected in August 1992 and used for all

Figure 1. Map of the lower Chesapeake Bay depicting the sites where mummichog and sediments were collected. AW: contaminated tidal creek adjacent to Atlantic Wood Industries, Inc. CI: reference site adjacent to Catlett Island. KC: secondary reference site in King Creek. JB: Jordan Toll Bridge.


NAVAL
SHIPYARD
ELIZABETH RIVER
AW
JB
NAVAL
SHIPYARD
YORK RIVER
CI
KC
CHESAPEAKE
BAY
JAMES RIVER
37 N
NORFOLK
ELIZABETH RIVER
76 W

preliminary and final static exposure studies (May-September 1993).

Prior to the flow-through experiment, a stock suspension of each sediment was prepared by mixing approximately two liters of wet sediment with 1-um filtered York River water to reach a dry weight concentration of 0.16 to 0.20 g/ml. Each suspension was homogenized using a plastic propeller connected to an overhead stir motor (Model 102, Talboys Engineering Corp., Emerson, NJ). The actual sediment concentration of each stock was determined from five 5-ml samples that were dried overnight at 60°C. The stock suspensions were stored tightly covered at 10°C when not in use.

For the preliminary static experiments, the AW and CI sediments were stirred manually and small amounts removed as needed. For the final static experiments, the sediments were stirred again and then small lots of each sediment type (1000 ml of CI sediment; 500 ml of AW sediment) were sieved through a 1-mm wire screen and stored in tightly covered glass jars at 10°C. These portions served as stock sediments for all subsequent static exposure studies. The dry weight/wet weight ratio for each stock was determined from aliquots of wet sediment that were weighed, dried overnight at 60°C, and then weighed again.

A creosote-amended sediment stock was prepared for use in one of the static exposure studies. A working solution

of creosote was made by dissolving 56 mg of creosote (marine grade, coal-tar distillate, Koppers Industries, Pittsburgh, PA) in 50 ml of acetone. 13.5 ml of this solution was mixed with 7.6 gm of oven-dried CI sediment and placed under a fume hood to allow the acetone to evaporate. When dry, the sediment was stored in a tightly covered glass jar at 10°C until needed. The quantities of creosote and sediment used were chosen to approximate the amount of total PAH (2 mg/gm dry weight) found in AW sediment used in earlier studies.

Embryos

Adult *Fundulus heteroclitus* were collected from each site using baited minnow traps. The fish were transported to the laboratory in aerated coolers and then moved to large tanks with flow-through sand-filtered York River water. They were fed twice daily with a mixture of Tetramarin and freeze-dried krill. Fish were collected in late June 1992 for the flow-through experiment, and then at various times between April and August 1993 for the static experiments.

The embryos needed for each experiment were obtained by *in vitro* fertilization in the laboratory following the protocol of Armstrong and Child (1965). Eggs were stripped manually from gravid females into glass fingerbowls containing 1-um filtered seawater, and mixed with sperm obtained by macerating testes in filtered seawater. After

30-60 minutes, the sperm suspension was rinsed from the eggs and replaced with fresh filtered seawater. The embryos were allowed to develop in an incubator (the temperature varied by experiment) until they reached the appropriate stage for exposure (which also varied by experiment; determination of embryonic stages was based on descriptions by Armstrong and Child 1965). At that time, unfertilized eggs and malformed embryos were discarded before the remaining embryos were randomly assigned to the appropriate treatments.

For all experiments except those involving hybrid embryos, eggs from several females were pooled and fertilized with sperm from several males. This was done in an attempt to obtain groups of embryos that were representative of the whole populations being studied. For the hybrid studies, however, it was necessary to test the offspring of individual pairs of fish in order to analyze the pattern of inheritance of the resistance trait.

One potential problem with pooling gametes from several fish was that all fish probably did not contribute equally to the pools of fertilized eggs. Some females provided more eggs than others, and some eggs may have been more easily fertilized than others. Similarly, some males may have provided more active sperm than others. However, as described in the Results of Experiments III and IV, there was little variation in response among batches of embryos from individual pairs of AW or CI fish (especially when

mortality was used as an end point). Thus, even if the pools of fertilized eggs were dominated by gametes from certain fish, the observed responses still should have been representative of the populations being examined.

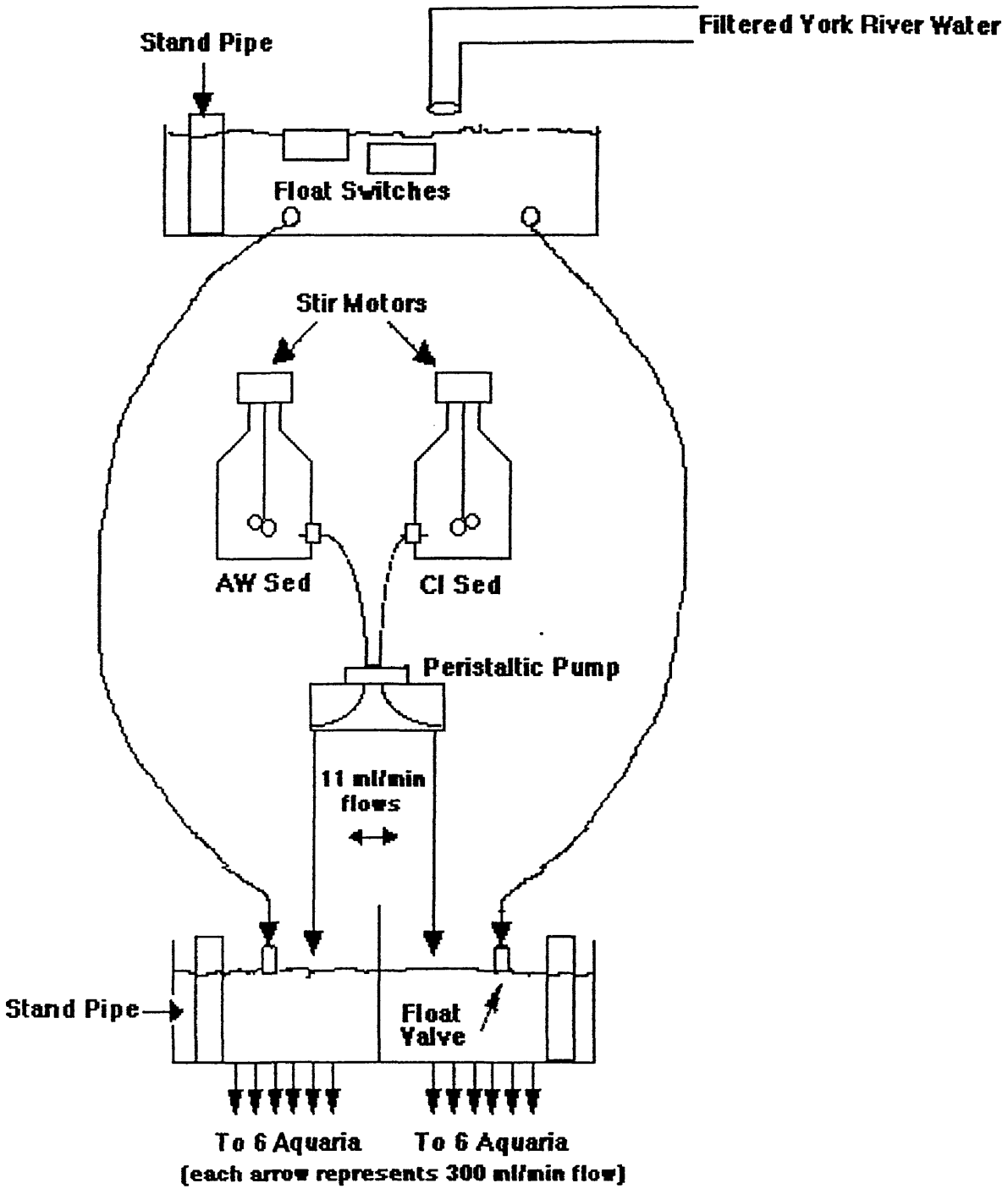
Experimental Procedure--Flow-Through System

A preliminary experiment was conducted to evaluate the differential response of CI and AW embryos to AW sediment and to determine the feasibility of exposing embryos to sediment in a flow-through system designed to deliver water and suspended sediment to a series of aquaria. Ideally, if this type of system was found to perform adequately, then it could be used in future exposure studies with larvae and adult fish as well.

The flow-through system used (Figure 2) was based on a design developed and tested at the Virginia Institute of Marine Science by Sved (1991). Certain modifications to this design were made to meet the specific needs of this study and to compensate for problems that arose during testing.

Working suspensions of each sediment type (AW and CI) were prepared in 20-L polycarbonate bottles covered with black plastic. Each bottle had a hole drilled in the side near the bottom, and a glass tube fitted through a stopper in the hole for withdrawal of the sediment suspension. Each

Figure 2. Schematic of the flow-through system used to deliver water and suspended sediment to a series of aquaria for a preliminary embryo exposure experiment (modified from Sved 1991).


Filtered York River Water
Stand Pipe
Float Switches
Stir Motors
AW Sed
Cl Sed
Peristaltic Pump
11 ml/min
flows
Stand Pipe
Float
Valve
To 6 Aquaria
To 6 Aquaria
(each arrow represents 300 ml/min flow)

bottle was filled with a mixture of the appropriate sediment stock suspension and filtered York River water. The amount of stock suspension used was calculated to yield a final sediment concentration of 20 mg/L in the aquaria based on the set flow rates (see below). The working sediment suspensions were renewed daily and were stirred continuously with stainless steel propellers driven by overhead motors (Model D226, Fasco Distributing Co., Ozark, MO) at a speed sufficient to keep the sediment from settling.

The sediment suspensions were pumped into two glass mixing chambers at a rate of 11 ml/min using a peristaltic pump (Model 1203, Harvard Apparatus, South Natick, MA). Filtered York River water was pumped into a vigorously aerated head tank, which was fitted with two float switches to control water depth. When the water level dropped below the lower switch, the pump was activated; when the level reached the upper switch, the pump was deactivated. If water pressure dropped so that a minimal depth could not be maintained, all power would have been interrupted and the system would have shut down. Water from the head tank flowed to the glass mixing chambers through float-controlled needle valves, used to maintain a constant hydrostatic pressure in each chamber.

Each chamber drained through six holes in the bottom which were fitted with pipette tips, each calibrated to give a flow rate of 300 ml/min each. The suspended sediment

solution then flowed through silastic and glass tubing to a series of twelve 10-gallon glass aquaria. The aquaria were connected to external standpipes via glass siphons to maintain a constant water volume of approximately 32 liters each. For the embryo exposures, an incubation cup consisting of a cylinder of 1-mm Nitex screen fitted over a glass petri dish bottom was placed on a stand in each of the aquaria, directly under the sediment inflow.

The embryo exposure experiment began on 28 July 1992 and ran for seven days. There were four treatments with three replicates each, randomly assigned among the twelve aquaria: 1) AW embryos on AW sediment; 2) AW embryos on CI sediment; 3) CI embryos on AW sediment; and 4) CI embryos on CI sediment. After *in vitro* fertilization, the embryos were allowed to develop to the four-cell stage before being randomly placed in the appropriate incubation cups (30 embryos/replicate). The embryos were examined daily under a dissecting microscope; dead embryos were counted and removed. Temperature, salinity, pH, dissolved oxygen, and flow rates were measured daily in each aquarium. In addition, a 500-ml water sample was collected daily from each aquarium, filtered, dried and weighed to determine suspended sediment concentration. On Day 7, the embryos were transferred to glass fingerbowls containing filtered York River water, placed in a 27°C incubator, and monitored until hatch.

Several difficulties were encountered in setting up this system and in conducting the exposure test. Some of these problems involved the malfunction of various components of the system. For example, the motor-driven propellers used to stir the working sediment suspensions quit on occasion, the peristaltic pump lines clogged, and the needle float valves did not maintain a constant water level. In addition, there were unexplained losses of sediment in the system. This made it necessary to more than double the calculated amount of sediment in the working suspensions in order to maintain an approximate final concentration of 20 mg/L in the exposure aquaria. The sediment losses could have been due to the settling that was observed in the pump lines, in the outflow tubes leading to the aquaria, in the mixing chambers, and in the polycarbonate bottles. And finally, the sediments and water did not appear to mix uniformly in the mixing chambers, leading to variability in the delivery of sediments to the aquaria.

Given these problems, and the general difficulties involved in maintaining such a complex system for extended periods of time, it was decided to develop a simpler static method for the subsequent embryo exposure studies.

Experimental Procedure--Static System

Based in part on methods described by Weis and Weis (1974; 1977a,b) and Sharp and Neff (1985), a system was devised for the static exposure of embryos to sediments. This exposure system consisted of a series of glass fingerbowls filled with mixtures of sediment and filtered seawater. Embryos were assigned randomly to these treatment bowls, which were covered with aluminum foil and then placed in a constant temperature incubator with a light:dark cycle of 14h:10h. The exposure tests ran for 96 hours, after which the sediments were removed from the bowls and replaced with clean filtered seawater. The bowls were returned to the incubator, and the embryos were monitored until all had hatched or died (approximately four weeks). Throughout each experiment, the embryos were examined daily under a dissecting microscope; the approximate stage of development (as described by Armstrong and Child 1965) was ascertained, any malformations or abnormalities were noted, and mortalities and hatched larvae were counted and removed as they occurred.

Two preliminary experiments were conducted to determine the optimal conditions for the final exposure studies. Details of these experiments are provided below.

Preliminary experiments The first trial of the static

exposure system was conducted in May 1993. The main purpose of this experiment was to determine the appropriate volume of AW sediment necessary to discriminate between resistant and susceptible embryos.

Four sediment treatments were tested in this study:

1) CI sediment: 20 ml of wet sediment and 55 ml of filtered seawater;

2) AW sediment (low volume): 15 ml of wet sediment and 60 ml of water;

3) AW sediment (high volume): 25 ml of wet sediment and 50 ml of water; and

4) AW sediment (high volume) with daily water changes.

Fifty KC and AW embryos were exposed to each treatment after 24 hours of development in a 20°C incubator (developmental stage was not recorded). All of the KC embryos exposed to AW sediments exhibited cardiac malformations and died within eight days. The exposed AW embryos also developed cardiac defects and died, but this occurred somewhat later than in the KC embryos.

Based on these results, a second experiment was designed to test lesser amounts of AW sediment. In addition, the incubator temperature was increased to 25°C to speed embryonic development, and a specific developmental stage was chosen for the start of the test. Given that Weis and Weis (1977a) had found the early blastula stage of *F. heteroclitus* to be the most sensitive to mercuric chloride

and considering the ease with which the blastula stages can be distinguished from earlier and later stages, it was decided to use mid-blastula embryos (stage 11-12) in all subsequent experiments.

Three sediment treatments were tested in this study:
1) CI sediment: 4 gm wet sediment and 50 ml of filtered seawater;
2) Low AW sediment (50%): 2 gm wet AW sediment, 2 gm wet CI sediment, and 50 ml of water; and
3) High AW sediment (75%): 3 gm wet AW sediment, 1 gm wet CI sediment, and 50 ml of water. Water was changed daily in all treatments.

Twenty KC and AW embryos were exposed to each treatment after they had reached stage 11. Again, all of the KC embryos exposed to AW sediments exhibited cardiac malformations and died within six days. Some of the exposed AW embryos also developed cardiac defects and died, but this occurred a few days later than in the KC embryos. In contrast to the previous experiment, over half of the AW embryos in the low AW treatment and 10% of those in the high treatment survived and hatched successfully.

Given these results, it was decided to begin the final exposure studies using amounts of AW sediment somewhat lower than those tested in this experiment. In addition, the incubator temperature was increased slightly to 27°C, since embryos from the flow-through experiment had developed

successfully and relatively rapidly at that temperature.

Experiment I This experiment was designed to address the first two research objectives, as well as to provide some insight into the third objective. Briefly, these objectives were to evaluate the differential resistance exhibited by AW and CI embryos when exposed to various amounts of AW sediment; to describe the effects of AW sediment on CI embryos; and to determine if the observed resistance could be due to genetic adaptation.

Twelve treatment combinations were tested in this study: AW and CI embryos were exposed to filtered seawater, CI sediment, low, medium and high amounts of AW sediment, and creosote-amended CI sediment (Table 1). The seawater controls were included to determine if exposure to sediment in any form had an effect on embryonic development and survival. The three levels of AW sediment were chosen to ascertain the amount of AW sediment that would best discriminate between resistant and susceptible embryos. The creosote treatments were included to determine if creosote could serve as an acceptable toxic substitute for the complex mixture of contaminants in AW sediment. If feasible, the use of creosote-amended sediment rather than AW sediment in subsequent exposure studies would increase the replicability of the experimental results. Creosote was chosen as a test substitute because the PAH which appear to

Table 1. Experiment I Treatments

TREATMENT	FILTERED SEAWATER	CI SEDIMENT (wet wt.)	AW SEDIMENT (wet wt.)	CREOSOTE-AMENDED SED (dry wt.)
H2O	50 ml			
CI	50 ml	4.0 gm		
CRE	50 ml	3.0 gm		0.28 gm*
LOW	50 ml	3.5 gm	0.5 gm	
MED	50 ml	3.0 gm	1.0 gm	
HIGH	50 ml	2.5 gm	1.5 gm	

* The creosote-amended sediment was designed to contain approximately the same concentration of PAH as AW sediment. Thus, 0.28 gm dry weight of this sediment should be equivalent to 0.28 gm dry weight of AW sediment, which corresponds to 1.0 gm wet weight (as was used in the medium AW treatment).

be the major contaminants at the AW site are likely to be derived from runoff or spills of creosote from the adjacent wood treatment facility. In fact, Buchman et al. (1992) found the PAH composition of sediments near AW to be similar to that found in creosote. However, there did appear to be some loss of the lighter PAH with a corresponding increase in the relative amounts of heavier PAH in the AW sediment as compared to creosote, which could be attributed to the effects of weathering and other physical processes at the AW site.

AW and CI embryos were obtained using gametes from eight females and four males from each population. These females were maintained in two aquaria, apart from the other laboratory fish, to provide eggs for Experiment II. While the embryos were developing in the 27°C incubator, 36 treatment bowls were prepared (12 treatments x 3 replicates each). A replicate sample of each sediment treatment was prepared at the same time and frozen for future chemical analysis. When the embryos reached stage 11 (approximately), they were randomly assigned to treatment bowls in groups of 25 and returned to the incubator. Water was changed daily in all bowls during the 96-hour exposure period. Samples of five embryos from one replicate per treatment were examined daily under a dissecting microscope as described earlier.

At the end of Day 4, the embryos were removed from the

sediments. On Day 5 and again on Day 10, they were screened for cardiac anomalies, using a slightly modified version of the cardiovascular index (CVI) developed by Weis et al. (1981a,b). In this index, 0=normal heart; 1=slight defect in structure or function; 2=tube heart with some degree of chamber development and a definite pulse; 3=tube heart with no chamber development and no true pulse (although there is some "ebb and flow" movement); and 4=beating tissue but no heart structure. This index was chosen because severe cardiac malformations had been observed in reference embryos exposed to AW sediment in the preliminary studies. The experiment was terminated after one month, when nearly all of the embryos had hatched or died.

Experiment II This experiment was designed to address the third research objective; i.e., whether or not the resistance exhibited by AW embryos could be due to genetic adaptation rather than physiological acclimation. The demonstration of increased toxicity resistance in AW embryos in the earlier experiments suggested that tolerance is heritable, since the embryos had never been exposed to contaminants directly and thus should not have been physiologically acclimated to them. However, there is the possibility that the eggs could have become acclimated through indirect exposure while developing in the female fish. This possibility was minimized in the earlier

experiments by keeping the fish in clean seawater for a depuration period before stripping them of eggs. Nevertheless, as a further test of the acclimation hypothesis, Experiment II was conducted using eggs obtained from some of the same female fish which had provided eggs for Experiment I. These fish had been held in clean seawater for the seven weeks between the two experiments. If the resistance was due to indirect physiological acclimation, then it would be expected that the embryos obtained for the second experiment would be less resistant than those obtained for the first experiment.

Four treatment combinations were tested in this study: AW and CI embryos were exposed to CI sediment and to the low amount of AW sediment (as described in Experiment I). This dose of AW sediment was found to be appropriate for distinguishing between resistant and susceptible embryos (as explained in Experiment I Results). AW embryos were obtained using gametes from two females and two males; CI embryos from two females and three males. There were two replicates of each AW sediment treatment and one replicate of each CI sediment treatment (total of 6 treatment bowls). As before, a sample of each sediment treatment was frozen for chemical analysis. A total of 161 CI embryos were obtained and randomly divided among three treatment bowls. Only 67 AW embryos were obtained; 25 were assigned to each AW sediment bowl and the remaining 17 were placed in the CI

sediment bowl. The experiment was conducted as described above for Experiment I.

Experiments III and IV These experiments were designed to address the final research objective, as well as to provide further insight into the question of genetic adaptation versus physiological acclimation. Briefly, the overall goal of these studies was to document the inheritance of the resistance trait in purebred AW and CI embryos as well as in hybrid AW x CI embryos.

For these experiments, batches of embryos obtained from individual pairs of fish were each divided into two groups and exposed to CI and AW sediments (the same low dose used in the previous two experiments). In Experiment III, eight batches of hybrid embryos were obtained by crossing CI females with AW males; in Experiment IV, the reciprocal crosses were performed (AW females with CI males). The sexes of the resistant and susceptible parent fish were reversed in these two experiments in order to ascertain whether or not the resistance trait showed any type of sex linkage.

In each experiment, purebred batches of embryos also were obtained from four pairs of AW fish and four pairs of CI fish. These embryos were included to determine if there was any variability in the resistance or susceptibility of embryos within these fish populations which may have been

masked in the previous experiments by using pooled gametes. In addition, the responses of these embryos provided benchmarks of the degree of resistance or susceptibility of each parental strain under the specific exposure conditions of each experiment.

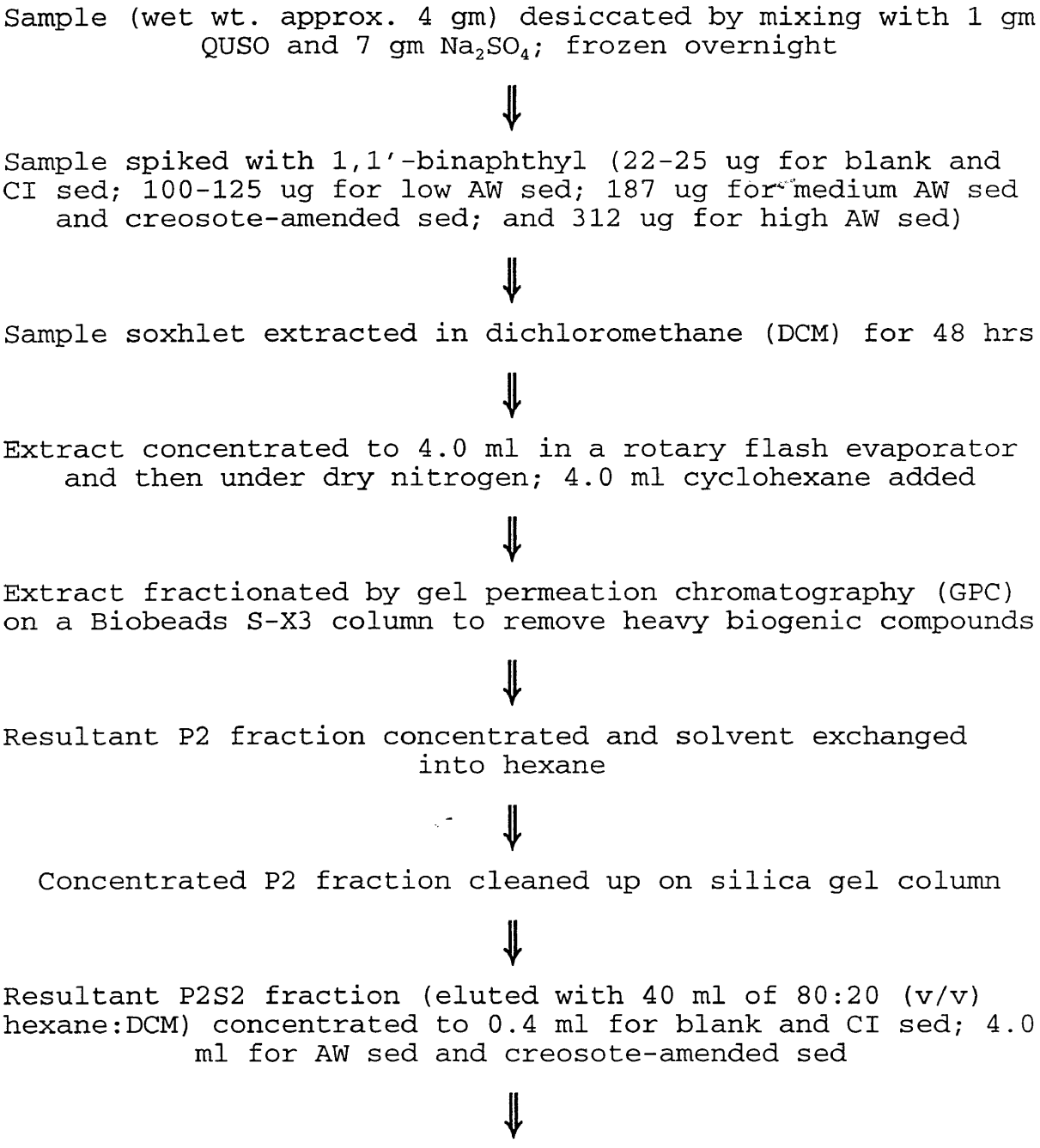
The number of embryos in each batch ranged from 40 to 335. In each case, the batch was split somewhat unevenly so as to place more embryos in the AW treatments (used to assess resistance) and fewer embryos in the CI treatments (used to assess overall viability of each batch). The experiments were conducted as described above for Experiment I.

Chemical Analyses

Given that PAH appear to be the major contaminants at the AW site, the sediment samples from all experiments were analyzed for total PAH to provide an index of the sediment contamination level. These analyses were conducted following the standard protocol used at the Virginia Institute of Marine Science (VIMS) for hazardous organic chemicals (Bieri et al. 1986). Details of the analytical procedures are shown in Figure 3.

Briefly, sediment samples were desiccated, spiked with an internal standard, and soxhlet extracted for 48 hours with dichloromethane. The resulting extracts then were

Figure 3. Analytical Procedure for Sediment Samples



fractionated by gel permeation chromatography (GPC) to remove high molecular weight biogenic compounds, and aromatic fractions were isolated by silica gel chromatography. These fractions were analyzed by capillary gas chromatography with flame ionization detection (GC/FID).

Identifications of individual compounds were based on retention times relative to known standards injected on the same day as each group of samples. Quantification of total resolved PAH in each sample was based on the internal standard, under the assumption that most PAH are recovered and detected with the same efficiency and sensitivity. Since it was impossible to determine wet weight/dry weight ratios for each sample (due to the small amount of sediment), PAH concentrations were calculated using the wet weight/dry weight ratios of the sediment stocks.

Statistical Analyses

Embryo responses to the various treatments were assayed using mortality and cardiovascular malformations as toxicity end points. For these experiments, mortality was defined as the total number of dead embryos, unhatched (deformed) embryos, severely deformed larvae, and dead larvae observed by the end of the experiment. As described earlier, embryos were screened for cardiovascular defects on Day 5 and Day 10 of each experiment and assigned cardiovascular index (CVI)

values ranging from 0 to 4.

The mortality data were analyzed using chi square tests (Fleiss 1981; Gad and Weil 1989). First, the frequencies of mortality in all replicates of a treatment were compared, and if no significant differences were observed, the data from all replicates were pooled for comparisons with other treatments. If the replicates were found to differ significantly, then further statistical analysis was not attempted and the treatments were compared qualitatively.

The data on cardiovascular malformations also were analyzed using chi square tests. These data consisted of the frequencies of embryos within each of the five CVI categories. Unfortunately, chi square analysis using all five categories was not possible in most cases because the frequencies in some of the categories were near zero. This resulted in several expected frequency values that were less than one, which caused the chi square test to be unreliable. In order to avoid this difficulty, the five categories were combined to form two larger categories. Embryos with a CVI value of 0 or 1 were classified as normal to slightly abnormal, while those with CVI values of 2-4 were classified as severely abnormal. Although it is clear that combining categories in this manner resulted in some loss of information, statistical analysis would not have been possible otherwise. The more detailed CVI data were retained in the graphs, which presented average CVI values

per treatment and per replicate.

Once the data had been classified into the two general categories, chi square analysis proceeded essentially as described above for the mortality data. In this case, the frequency of severely abnormal embryos was the parameter used for comparisons among replicates and treatments.

All statistical analyses were conducted using Statistix^{R} software (Analytical Software, St. Paul, MN).

RESULTS

Preliminary Flow-Through Experiment

Water quality parameters (temperature, salinity, pH, and dissolved oxygen) remained fairly constant in all twelve aquaria throughout the seven day exposure period (Table 2). The rate of flow of the suspended sediments to each aquarium appeared to be fairly steady as well, with much less than a 10% variation over time and among aquaria (Table 2). The concentrations of suspended sediments were somewhat more variable, however, both over time and among aquaria (Table 2). Most of the variation among aquaria was found in the AW sediment treatments, with mean concentrations ranging from 19.3 to 25.4 mg/l. The overall average concentration in the six aquaria receiving AW sediment (22.0 mg/l) did not differ significantly from the overall average CI sediment concentration (21.1 mg/l) in a t-test ($p=0.43$), but the variances of the two groups did differ significantly ($p=0.01$).

The variability in suspended sediment concentrations could have been due to the difficulties with the sediment delivery system that were discussed above in the Methods section. In addition, measurement error could have

Table 2. Water Quality and Suspended Sediment Parameters for the Preliminary Flow-Through Experiment

TREATMENT		TANK	WATER QUALITY[a]				SUSPENDED SED	
EMB	SED		TEMP (C)	SAL (ppt)	PH	D.O. (mg/l)	FLOW[a] (ml/min)	CONC[b] (mg/l)
AW	CI	6	26.3 (.5)	22 (0)	7.76 (.09)	6.41 (.11)	297 (3)	22.1 (3.8)
AW	CI	8	25.8 (.5)	22 (0)	7.76 (.08)	6.47 (.07)	294 (4)	21.5 (2.3)
AW	CI	9	26.0 (.4)	22 (0)	7.76 (.08)	6.50 (.08)	302 (3)	21.3[c] (4.1)
CI	CI	2	26.1 (.5)	22 (1)	7.79 (.09)	6.53 (.13)	284 (2)	20.1 (2.6)
CI	CI	3	26.1 (.5)	22 (1)	7.78 (.09)	6.55 (.13)	290 (1)	20.1 (3.4)
CI	CI	7	26.1 (.5)	22 (0)	7.77 (.08)	6.51 (.10)	304 (2)	21.4 (3.1)
AW	AW	1	26.0 (.4)	22 (0)	7.75 (.09)	6.15 (.18)	313 (2)	23.5 (2.6)
AW	AW	10	26.0 (.4)	22 (0)	7.75 (.08)	6.36 (.09)	296 (4)	19.3[c] (1.8)
AW	AW	11	26.3 (.4)	22 (0)	7.73 (.08)	6.44 (.09)	300 (3)	24.0[c] (2.6)
CI	AW	4	26.3 (.4)	22 (0)	7.76 (.10)	6.54 (.15)	295 (3)	19.7 (3.2)
CI	AW	5	26.2 (.5)	22 (0)	7.75 (.08)	6.30 (.11)	300 (4)	20.2 (2.1)
CI	AW	12	26.3 (.4)	22 (0)	7.73 (.09)	6.43 (.08)	311 (5)	25.4[c] (3.0)

[a] Values are means of seven daily measurements; standard deviations are in parentheses.

[b] Values are means of six daily measurements; Day 7 concentrations were abnormally high (possibly due to procedural error) and thus were not included.

Values are means of five daily measurements; concentrations on Day 2 were not included because they were much higher than usual, due to the use of a new type of filter (see text).

contributed to the problem. As mentioned in Table 2, the new type of 0.45 um filter (47 mm, Type GN-6, Gelman Sciences, Ann Arbor, MI) that was used to filter water samples from tanks 9-12 on Day 2 was found to collect much more sediment than the filter type used previously (47 mm, Type GA-6, Gelman Sciences, Ann Arbor, MI). The results with the new filters were thought to be more realistic, so the amount of sediment in the working suspensions was reduced somewhat to achieve a final concentration of approximately 20 mg/l in the exposure aquaria. It is possible that this change of filter types during the experiment and the subsequent adjustment of sediment amounts in the working suspensions may have caused some of the variability seen in the final sediment concentrations.

Despite the technical problems encountered during this experiment, there were some promising results obtained from the embryo exposures. After approximately four days of exposure to AW sediment, the CI embryos exhibited abnormal cardiac development. The observed malformations were classified as tube heart with pericardial edema, based on the descriptions in Sharp (1991). By the fifth day, no pulse was visible in many of these embryos, but to avoid errors, they were not labeled mortalities immediately. After nine days it was apparent that all of these embryos were indeed dead. In contrast, CI embryos exposed to CI sediment and AW embryos exposed to either type of sediment

appeared to exhibit normal cardiac development. There were mortalities in each of these groups, but many of these could be attributed to unsuccessful fertilization (not detected due to the early stage of the embryos used in the experiment), experimental error in identifying dead embryos (especially in the early stages), and problems with the hatching protocol (which was modified for the static experiments). Notwithstanding these complicating factors, the preliminary mortality data (Table 3) were consistent with the hypothesis that AW mummichog embryos are resistant to the acute toxicity of local sediments.

Static Experiments

Chemical analyses Results of the PAH analyses of sediment samples from all static experiments are shown in Table 4. Total PAH per treatment bowl was included because it was felt that this provided a more reliable indicator of relative sediment contamination in these experiments than the more standard measure of total PAH concentration. The problem with the latter index was that it required a dry weight:wet weight ratio for each sample; however, in these experiments the samples were too small for those measurements to be made, so the weight ratios for the sediment stocks were used as approximate substitutes. This should have been a valid substitution, given that the same

Table 3. Mortality Data for the Preliminary Flow-Through Experiment

TREATMENT[a]		PERCENT MORTALITY[b]
EMBRYO TYPE	SEDIMENT TYPE	
AW	CI	32
CI	CI	12
AW	AW	26
CI	AW	100

[a] Each treatment consisted of three replicate aquaria with 30 embryos apiece, for a total n = 90.

[b] Values were determined by the total number of dead embryos in each treatment divided by the total number of embryos in each treatment.

Table 4. Total Resolved PAH in Sediment Samples from Static Experiments

EXPERIMENT	SED TYPE	TOTAL PAH CONC[a] (mg/gm dry wt)	TOTAL PAH/BOWL[b] (mg/trt bowl)
I	CI	BLD	0.01
I	CRE	0.07	0.10
I	AW-LOW	1.05	1.49
I	AW-MED	2.07	2.86
I	AW-HIGH	2.94	3.93
II	CI	BLD	BLD
II	AW-LOW	ND	ND
III	CI	ND	ND
III	AW-LOW	ND	ND
IV	CI	BLD	BLD
IV	AW-LOW	0.64	0.91

ND Not determined, due to procedural error which led to the loss of these samples.

BLD Below the limit of detection. Detection limit for total PAH concentration = 34.96 ng/gm dry weight; detection limit for total PAH/bowl = 51.48 ng/bowl.

Calculated as follows: 1) the actual amount of CI and AW sediment in each sample was determined based on the total sample wet weight and the expected ratios of sediment types in that sample; 2) a sample dry weight was calculated based on the amounts of each sediment type in the sample and the known dry weight:wet weight ratios for each sediment type (0.2785 for AW and 0.3682 for CI); and 3) the total resolved PAH in the sample was divided by the sample dry weight.

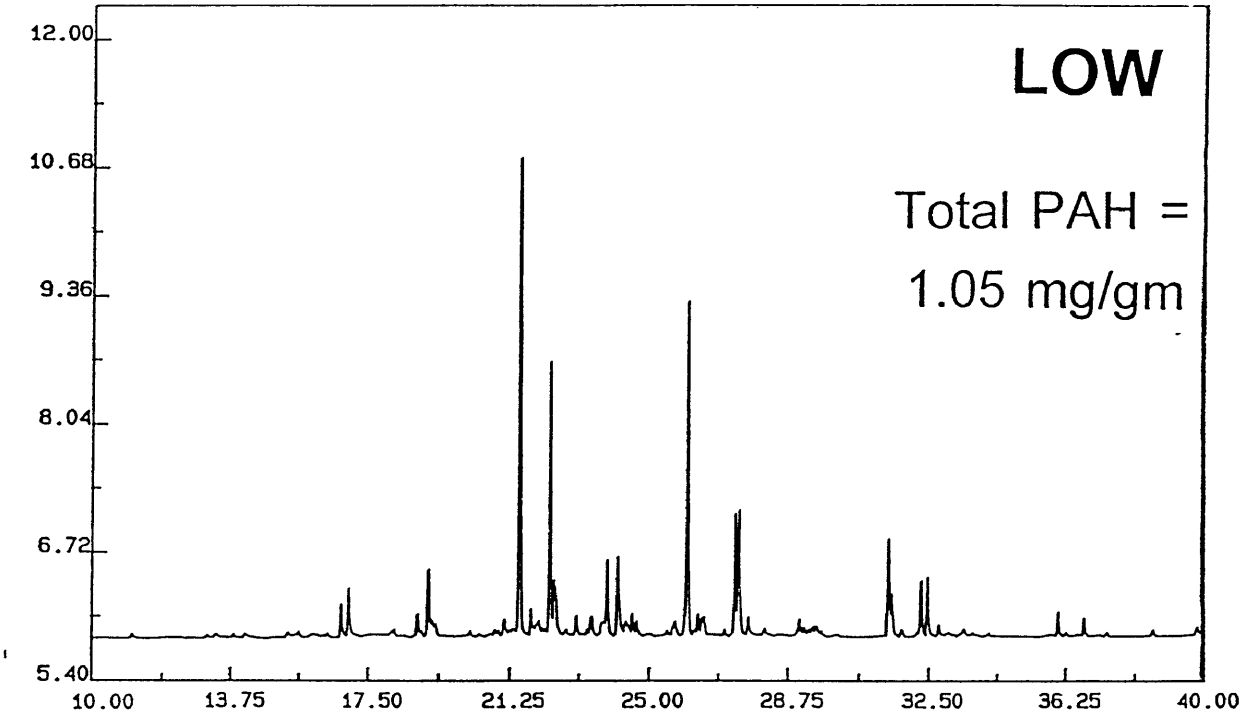
[b] Calculated as follows: the total resolved PAH per gram of sample (wet weight) was multiplied by the nominal amount of sediment in each treatment bowl (4.0 gm wet weight).

stocks were used in all experiments (which took place over a three month period), but it did introduce a possible source of error.

In all experiments, the AW sediment treatments contained orders of magnitude more PAH than the reference CI sediment treatments, which exhibited no detectable PAH. A representative GC/FID chromatogram obtained by analysis of a sample from an AW sediment treatment is shown in Figure 4.

In Experiment I, the total PAH in the AW treatments tracked fairly closely with the nominal relative amounts of AW sediment in each treatment. The medium dose (25% AW sediment) contained approximately 1.9 times the PAH of the low dose (12.5% AW sediment), and the high dose (37.5% AW sediment) contained approximately 2.7 times the PAH of the low dose and 1.4 times the PAH of the medium dose. Unfortunately, only one AW treatment sample from the subsequent experiments was analyzed, due to loss of the other samples. The amount of PAH in this sample (Experiment IV, AW-LOW) was somewhat lower than the amount of PAH in the Experiment I low dose, although the treatments were prepared similarly. This result raised the question of whether or not the AW sediment stock might have lost some potency over time. However, given the variability inherent in sampling and analysis, and the fact that only one replicate from each experiment was analyzed, the observed difference may not have been significant. In addition, a similar treatment

Figure 4. GC/FID chromatogram of the aromatic fraction of an extract of a sediment sample from Experiment I which contained the low dose of AW sediment.


LOW
Total PAH =
1.05 mg/gm
12.00
10.68
9.36
8.04
6.72
5.40
10.00
13.75
17.50
21.25
25.00
28.75
32.50
36.25
40.00

sample from a later related experiment was found to contain an intermediate amount of PAH (0.75 mg/gm or 1.07 mg/bowl), again suggesting that the AW stock had not lost significant amounts of PAH.

The creosote treatment tested in Experiment I contained much less PAH than expected (0.07 mg/gm as compared to the intended 0.5 mg/gm). This may have been due to flaws with the method used to add the creosote to the CI sediment, or it could have been due to analytical error. When the creosote-amended sediment sample was being concentrated on the rotary flash evaporator before GC analysis, it was allowed inadvertently to evaporate completely, which could have resulted in the loss of the more volatile PAH.

The AW sediment stock used in these experiments apparently was much more contaminated than that analyzed in some earlier studies (Vogelbein et al. 1990; Buchman et al. 1992). This result was not too surprising, given the variable nature of field sediments and the likelihood of there being "hot spots" of contamination scattered throughout the AW area. The specific PAH composition of the AW sediment was not analyzed, since total PAH was being used only as an index of the contamination level of each treatment.

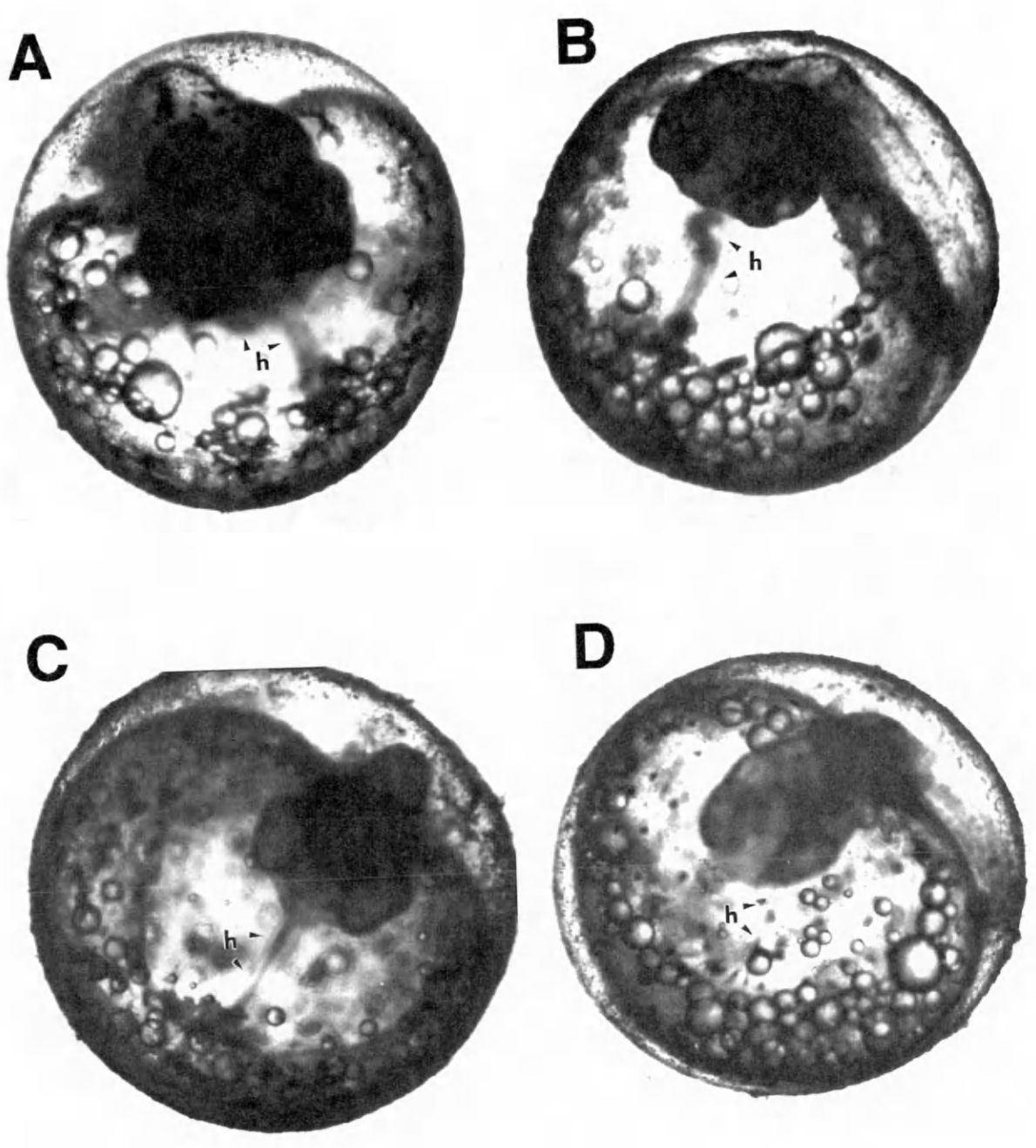
Developmental defects While embryos in the preliminary experiments were monitored for the development of any

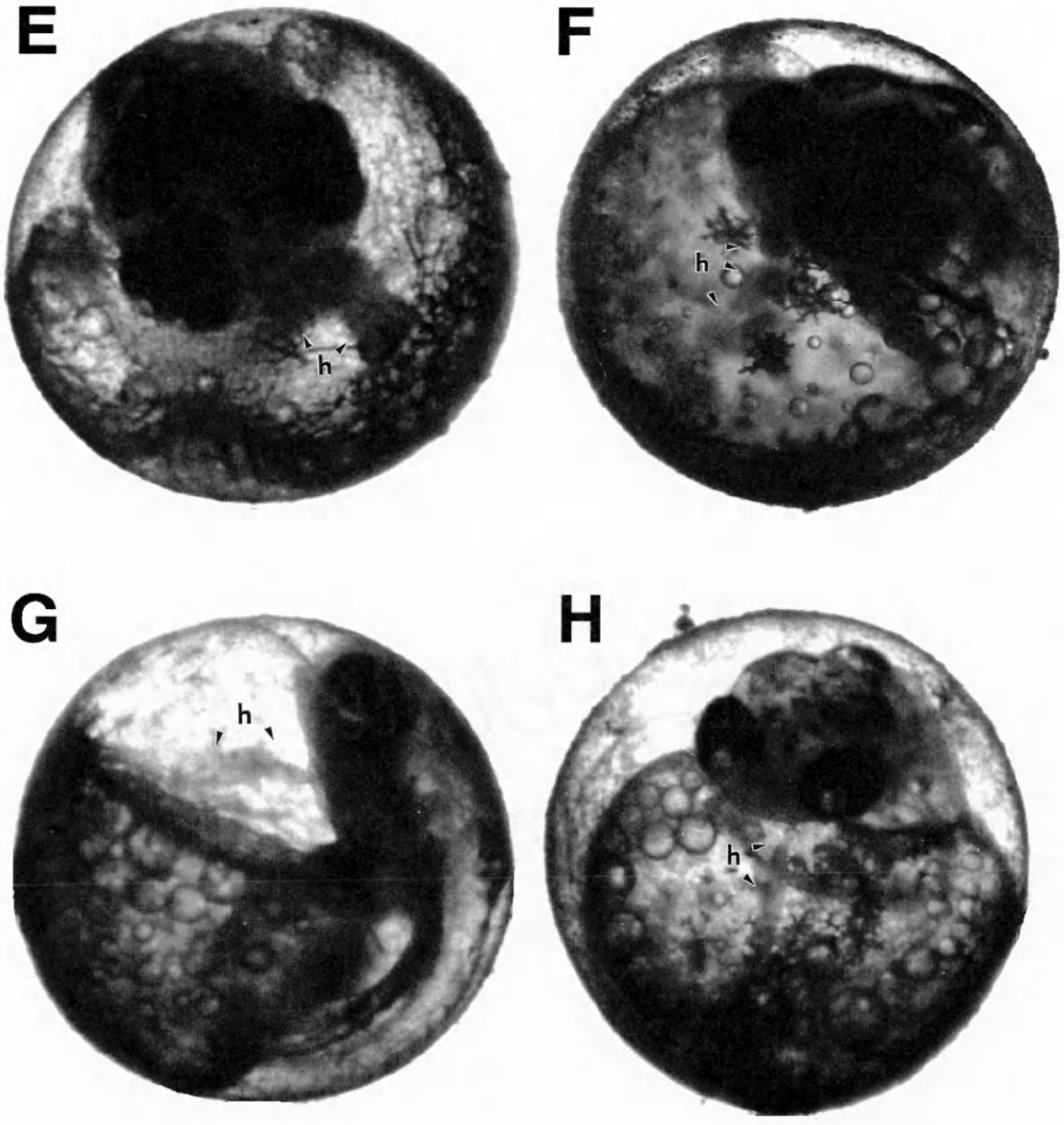
abnormalities, only cardiovascular defects were observed with any regularity. There were some instances of craniofacial or skeletal defects, but these were not very common and appeared to be fairly randomly distributed across treatments. Therefore, embryos in the subsequent experiments were screened only for cardiovascular abnormalities.

The types of cardiac malformations observed in susceptible embryos exposed to contaminated sediment in all experiments ranged from slight structural or functional defects to the absence of any discernible structure. The latter defect was observed only rarely, and did not appear to be related to contaminant effects. The most commonly observed defect was tube heart with pericardial edema (Figure 5C,D,G,H). In this case, the heart was a thin, weakly pulsating tube with no chamber development, stretching between the head and the yolk. Blood did not appear to move through the heart, although it did wash back and forth within the tube. Fluid filled the pericardial space, and static pools of blood often were seen within this space at the base of the heart. Growth of these embryos usually appeared to be arrested after the development of these abnormalities, although this effect was not monitored consistently. Affected embryos still exhibited spontaneous movements similar to those seen in normal embryos.

A somewhat less severe version of tube heart was

Figure 5. Examples of the types of cardiovascular defects observed in contaminant-exposed embryos (h = heart structures). A. Normal Day 6 embryo (CVI=0). B. Day 6 embryo exhibiting pericardial edema and a tube heart with some chamber development (CVI=2). C and D. Day 6 embryos exhibiting pericardial edema and severe tube hearts lacking chamber development (CVI = 3). E. Normal Day 9 embryo (CVI=0). F. Day 9 embryo exhibiting pericardial edema and a tube heart with some chamber development (CVI=2). G and H. Day 9 embryos exhibiting pericardial edema and severe tube hearts lacking chamber development (CVI = 3).


A
h
B
h
C
h
D
h


E
h
F
h
G
h
H
h

observed frequently as well (Figure 5B,F). In this case, the heart was usually a somewhat thicker tube which exhibited varying degrees of chamber development and a definite pulsing flow of blood through the chambers. Fluid still filled the pericardial space, but blood pooling was not as common.

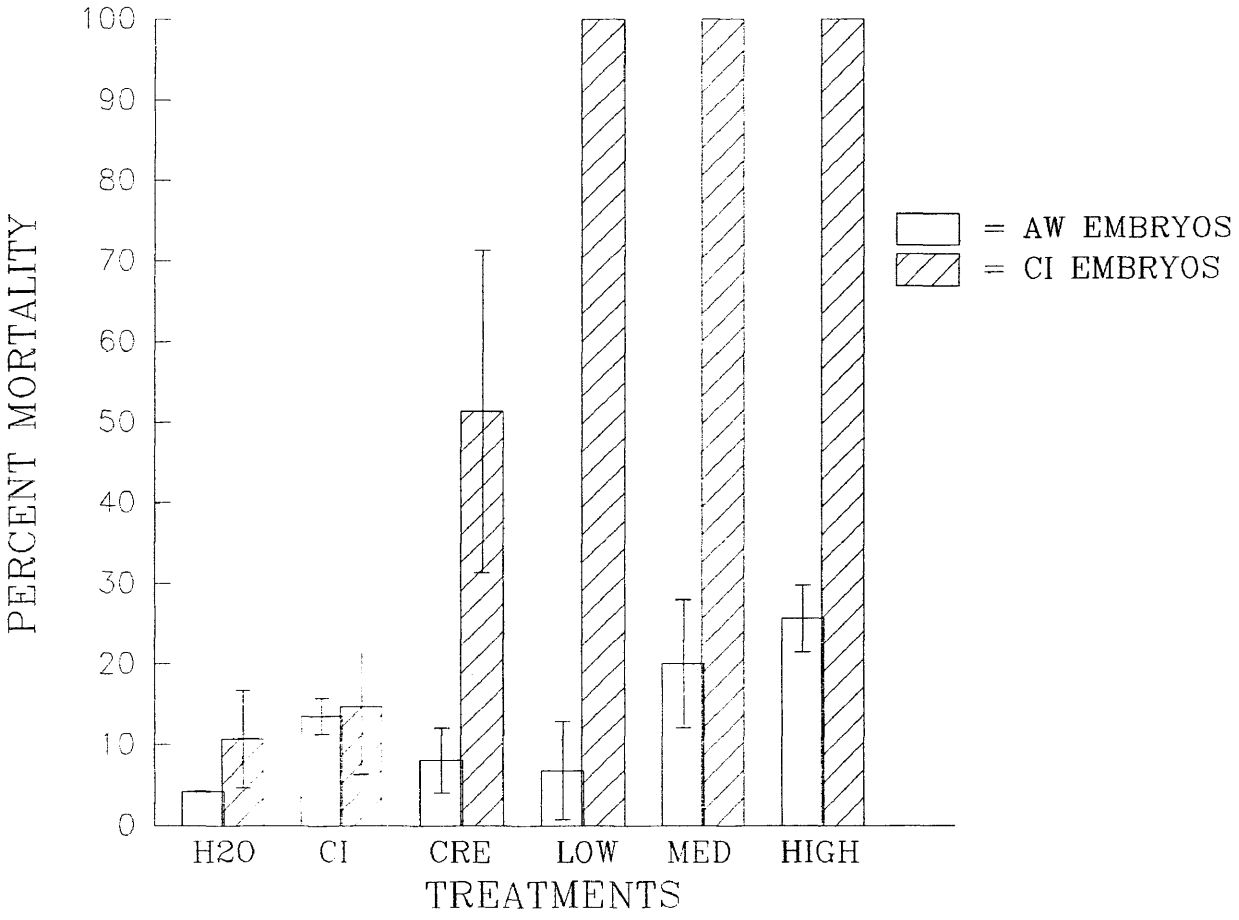
Experiment I mortality The responses of AW and CI embryos to the six sediment treatments in terms of mean percent mortality are shown in Figure 6. The CI embryos exhibited nominal mortality rates when exposed to water or CI sediment, approximately 50% mortality when exposed to creosote-amended sediment, and 100% mortality when exposed to any dose of AW sediment. The AW embryos, on the other hand, exhibited nominal mortality rates when exposed to water, CI sediment, creosote-amended sediment or the low dose of AW sediment. They exhibited somewhat increased mortality (20-25%) when exposed to the medium and high doses of AW sediment, but still much less than that seen with the CI embryos.

Chi-square analysis of the frequency of mortality in each treatment (using pooled values from three replicates) showed that AW and CI embryos responded similarly to the water and CI sediment treatments ($p=0.18$). Thus, it appeared that sediment alone did not have a significant effect on embryo mortality as compared to water.

Figure 6. Mean mortality rates for all treatments in Experiment I. The percent mortality in each replicate of a treatment was determined, and then these values were averaged to provide the mean mortality rate for that treatment. There were three replicates of each treatment. The vertical bars represent the standard deviations around the mean values (no variability was seen in the CI embryo/AW sediment treatments).

EXPERIMENT I

MORTALITY



Consequently, CI sediment was used as the sole control in subsequent experiments. These results also demonstrated that AW and CI embryos adapted equally well to the exposure conditions used in this study, with no inherent differences in their ability to survive in the control treatments.

Extreme differences in survival of AW versus CI embryos were seen in all of the AW sediment treatments. AW embryos exhibited much lower mortality rates than CI embryos in these treatments; differences which were shown to be very significant by chi-square analysis ($p<0.001$ in all cases). The frequency of mortality among CI embryos exposed to any of the AW sediment treatments was much higher than in those exposed to CI sediment; a difference which again was found to be very significant ($p<0.001$). Given these results, the low AW dose was used in all subsequent experiments, since it seemed to discriminate well between resistant and susceptible embryos.

The AW embryos did not exhibit complete toxicity resistance. Chi-square analysis comparing AW embryo mortality rates in all treatments found them to be similar among the four least contaminated treatments (water, CI sediment, creosote-amended sediment, and the low AW dose; $p=0.21$) and between the two most contaminated treatments (medium and high AW doses; $p=0.53$), but there was a significant difference between these two groups ($p<0.001$).

Analysis of the effects of creosote-amended sediment on

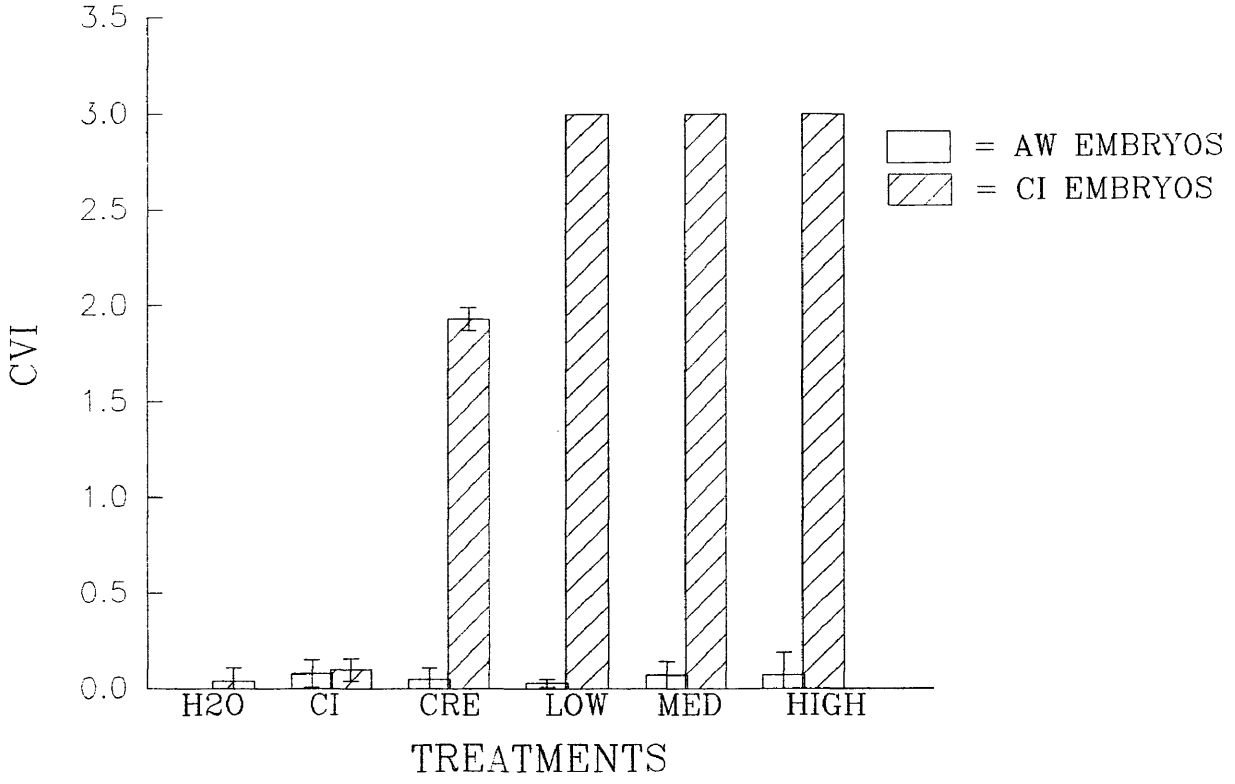
embryo mortality was complicated by the variability observed in the treatment exposing CI embryos to this sediment. Mortality rates in the three replicates of this treatment were found to differ significantly ($p=0.02$). These differences probably were due to inconsistencies in preparation of the sediment for each replicate, since the embryos were pooled before being placed randomly in each treatment and thus were unlikely to be the source of the variability. It is possible that the toxicity of this sediment was near the average threshold level required for CI embryo mortality, and thus even slight differences among replicates could have caused extreme differences in response. Similarly, the sediment toxicity was too low for the more resistant AW embryos to be affected, and thus there was little variability among replicates of that treatment. However, the results demonstrated that the amount of creosote used in this experiment was toxic to some proportion of CI embryos, while having no effect on AW embryos.

Experiment I cardiovascular abnormalities The responses of AW and CI embryos to the six sediment treatments in terms of cardiovascular defects are shown in Figure 7. On Day 5, the CI embryos exposed to water or CI sediment exhibited very low average cardiovascular index (CVI) values (0.04 and 0.10, respectively), which were

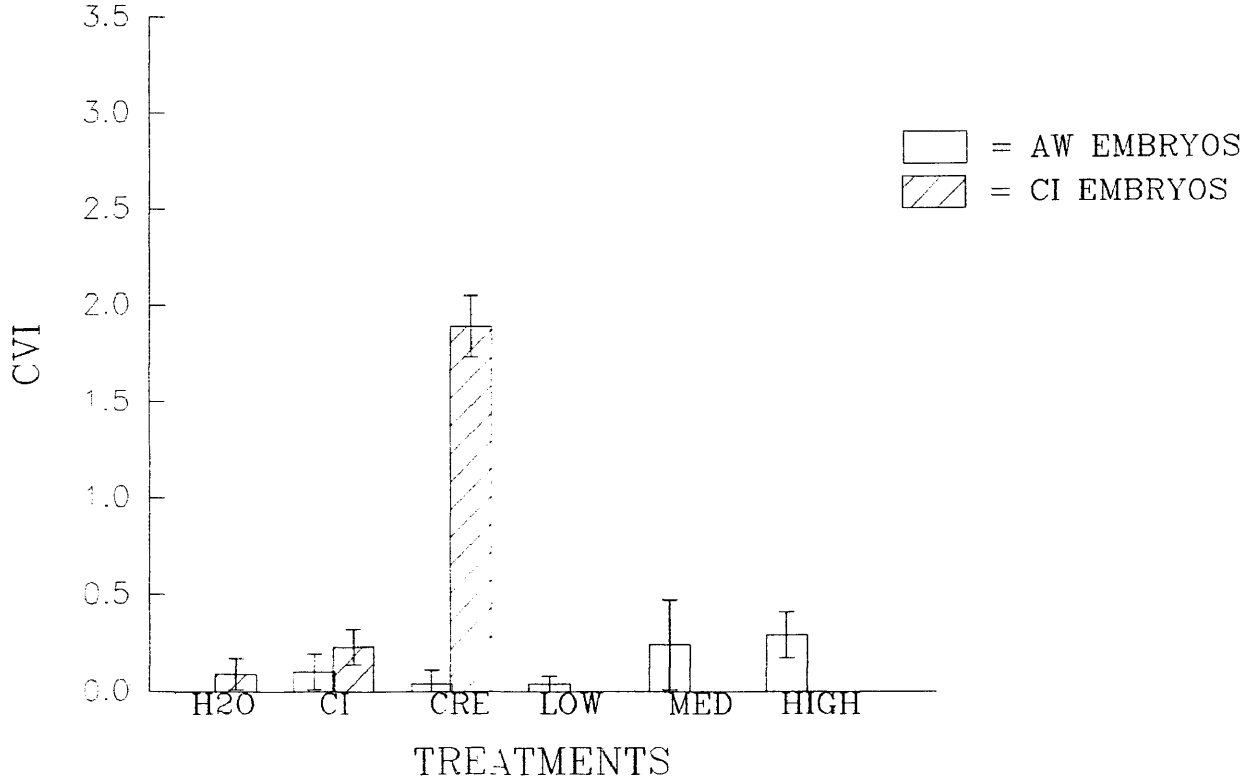
Figure 7. Mean cardiovascular index (CVI) values for all treatments in Experiment I. The mean CVI value for each replicate of a treatment was determined, and then these values were averaged to provide the overall mean CVI value for that treatment. There were three replicates of each treatment, except on Day 10, when all embryos in the CI embryo/AW sediment treatments were dead. The vertical bars represent the standard deviations around the means (no variability was seen in the CI embryo/AW sediment treatments on Day 5).

EXPERIMENT I

CARDIOVASCULAR ABNORMALITIES: DAY 5



CARDIOVASCULAR ABNORMALITIES: DAY 10



indicative of the relative lack of developmental abnormalities in these groups. CI embryos exposed to creosote-amended sediment or any dose of AW sediment exhibited much higher average CVI values (1.93 and 3.00, respectively), due to the greater frequency and severity of cardiac defects in these groups. In contrast, AW embryos exhibited low average CVI values (<0.10) in all treatments.

The results of screening for cardiovascular defects on Day 10 also are shown in Figure 7, although the usefulness of these results is limited somewhat by the number of mortalities that had occurred by that time. Again, AW embryos exhibited nominal average CVI values in all treatments (<0.30), although the values for the medium and high AW doses were slightly higher than on Day 5. The variances in these treatments were fairly large, however, and the differences can not be considered significant. As before, the CI embryos exhibited low average CVI values when exposed to water or CI sediment (0.09 and 0.23, respectively), and a much higher average value (1.89) when exposed to creosote. All of the CI embryos exposed to AW sediment were dead by Day 10, so no comparisons with the results of the other treatments were possible on that day. For that reason, statistical analyses were conducted using only the Day 5 CVI data.

Chi-square analysis of the frequency of severely abnormal embryos (CVI=2-4) in each treatment (using pooled

values from three replicates) showed that AW and CI embryos responded similarly to the water and CI treatments ($p=0.52$). Thus, sediment alone did not have an effect on the development of cardiovascular abnormalities in exposed embryos. In addition, the AW and CI embryos demonstrated no inherent differences in their ability to develop normally in control treatments under the exposure conditions used in this study.

In contrast, AW and CI embryos exhibited a clear disparity in their ability to develop normally when exposed to AW sediment. The frequencies of severe cardiovascular abnormalities in exposed AW embryos were much lower than those seen in CI embryos in all of the AW sediment treatments; differences which were highly significant by chi-square analysis ($p<0.001$ in all cases). The prevalence of severe abnormalities among CI embryos exposed to any of the AW sediment treatments was much higher than in those exposed to reference CI sediment; a difference which again was found to be very significant ($p<0.001$). These results supported the decision to use the low AW dose in all subsequent experiments, since it allowed discrimination between resistant and susceptible embryos using either cardiovascular defects or mortality as an end point.

The resistance demonstrated by AW embryos extended uniformly to all treatments. Chi-square analysis showed that the frequencies of severely abnormal AW embryos in all

sediment treatments did not differ significantly ($p=0.68$). These results indicated that even the high dose of AW sediment was not toxic enough to cause teratogenic effects in AW embryos that would be apparent by Day 5; however, this does not preclude the appearance of such defects at a later time. The observation of slightly higher average CVI values on Day 10 for AW embryos exposed to the medium and high doses of AW sediment (as compared to Day 5), although not statistically significant, suggested that these embryos may have had incipient defects which worsened over time.

The creosote-amended sediment appeared to have had an effect on CI embryos that was qualitatively similar to but quantitatively less than that elicited by AW sediment. The susceptible embryos developed cardiovascular defects that resembled those seen in CI embryos exposed to AW sediment, although not with the same degree of severity. Statistical analysis of pooled data was possible in this case due to the lack of variability among the three treatment replicates. Chi-square analysis showed the frequency of severe cardiovascular abnormalities to differ significantly between the creosote treatment and the low AW sediment treatment ($p<0.001$), as well as between the creosote treatment and the reference CI sediment treatment ($p<0.001$). In addition, AW and CI embryos exhibited significantly different frequencies of severe abnormalities when exposed to the creosote-amended sediment ($p<0.001$). These results indicated that creosote,

in the amount used in this experiment, was able to elicit a teratogenic response in susceptible CI embryos similar to that seen with AW sediment, while having no effect on AW embryos.

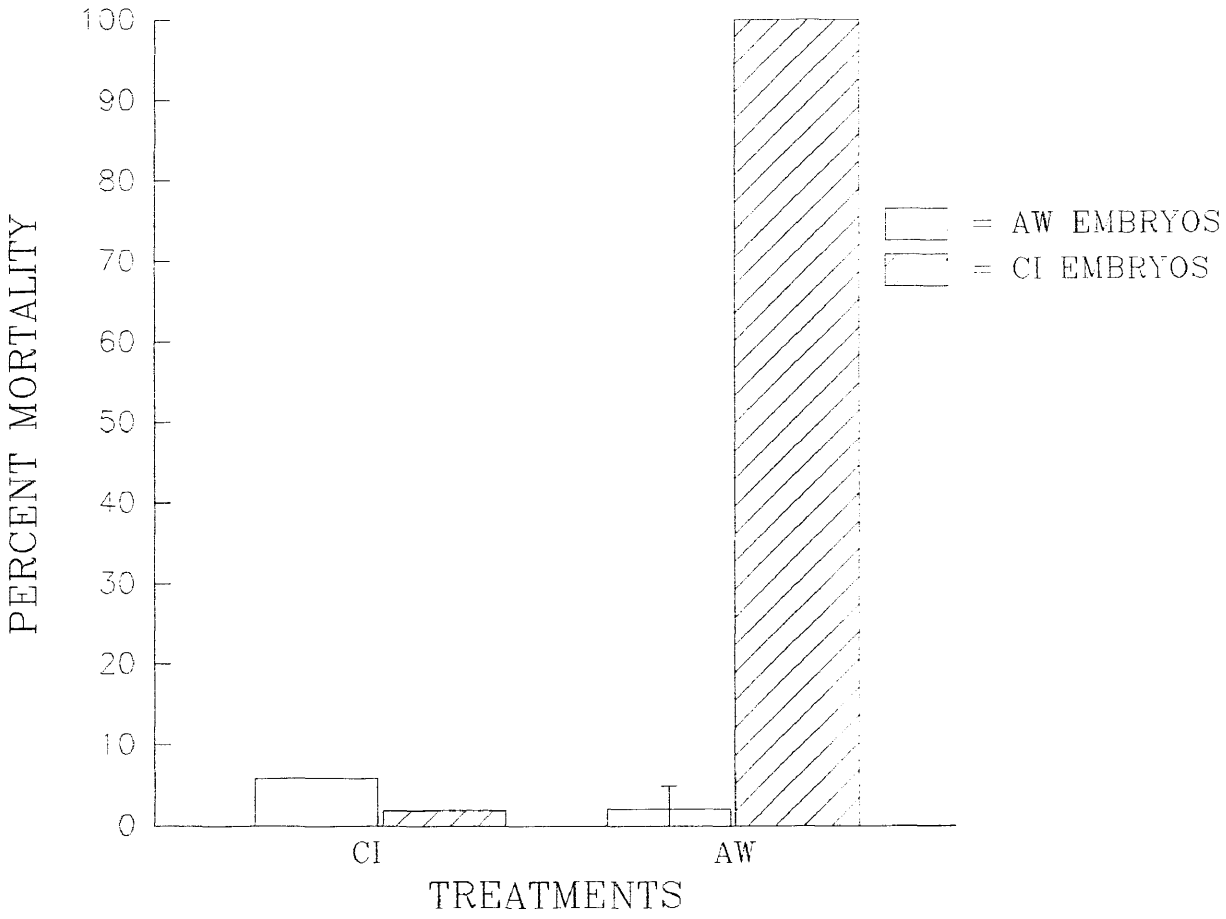
Experiment II mortality The average mortality rates of AW and CI embryos exposed to AW and CI sediment are shown in Figure 8. As explained in the Methods section, the relatively small number of eggs obtained from female fish saved from Experiment I allowed for only two replicates of each AW sediment treatment and one replicate of each CI sediment treatment. The small sample sizes limited interpretation of the results from this study, but still permitted the elucidation of general trends.

CI embryos exhibited a nominal mortality rate (1.9%) when exposed to CI sediment, but complete mortality (100%) when exposed to AW sediment. In contrast, AW embryos exhibited nominal mortality rates (<6%) in both treatments. Chi-square analysis of the frequency of mortality in each treatment (using pooled values from two replicates where appropriate) showed that AW and CI embryos responded similarly to the reference CI sediment ($p=0.98$), indicating that both groups of embryos adapted equally well to the experimental conditions. However, the two groups of embryos responded very differently to the contaminated AW sediment ($p<0.001$). The AW embryos were no more affected by the AW

Figure 8. Mean mortality rates for all treatments in Experiment II. The percent mortality in each replicate of a treatment was determined, and then these values were averaged to provide the mean mortality rate for that treatment. There was one replicate of each CI sediment treatment and two replicates of each AW sediment treatment. The vertical bars represent the standard deviations around the means (no variability was seen in the CI embryo/AW sediment treatment).

EXPERIMENT II

MORTALITY



sediment than they were by the CI sediment ($p=1.00$), while the CI embryos exhibited significantly higher mortality in the AW sediment treatment than in the CI treatment ($p<0.001$).

These results were very similar to those of Experiment I. In fact, a direct comparison of the responses of AW embryos exposed to the low dose of AW sediment in each experiment showed that the second batch of embryos actually had a lower average mortality rate (2.0%) than the first batch (6.8%), although the difference was not statistically significant ($p=0.43$). At the same time, the CI embryos exposed to the contaminated sediment exhibited 100% mortality in both experiments, which indicated that the sediment used in the second experiment was just as lethal to susceptible embryos as that used in the first study.

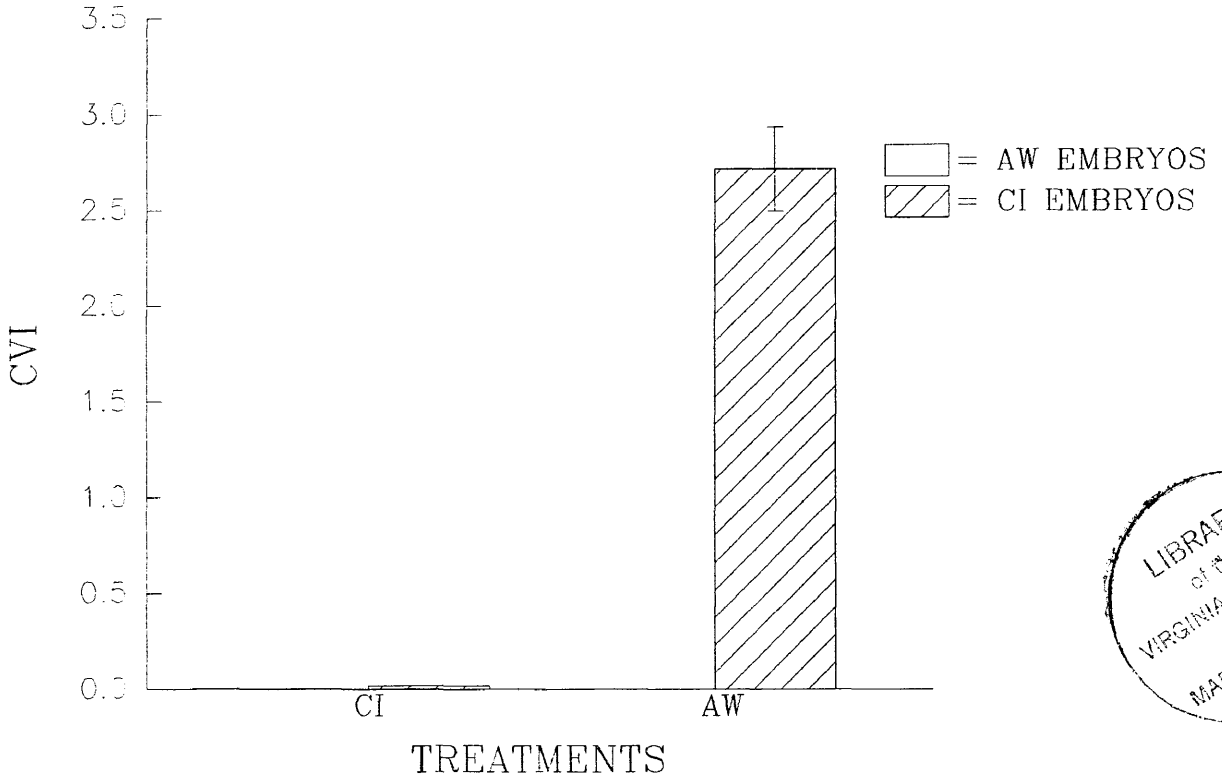
Experiment II cardiovascular abnormalities The average CVI values of AW and CI embryos exposed to AW and CI sediment are shown in Figure 9. On Day 5, the CI embryos exposed to CI sediment exhibited a very low average CVI value (0.02), while those exposed to AW sediment exhibited a very high average CVI value (2.72). In contrast, no cardiovascular abnormalities were apparent in the AW embryos exposed to either sediment (average CVI=0).

The Day 10 CVI values also are shown in the figure, although as before, the usefulness of these results is

Figure 9. Mean cardiovascular index (CVI) values for all treatments in Experiment II. The mean CVI values for each replicate of a treatment was determined, and then these values were averaged to provide the overall mean CVI value for that treatment. There was one replicate of each CI sediment treatment and two replicates of each AW sediment treatment. The vertical bars represent the standard deviations around the means (no variability was seen in the AW embryo/AW sediment treatment on either day or in the CI embryo/AW sediment treatment on Day 10).

EXPERIMENT II

CARDIOVASCULAR ABNORMALITIES: DAY 5



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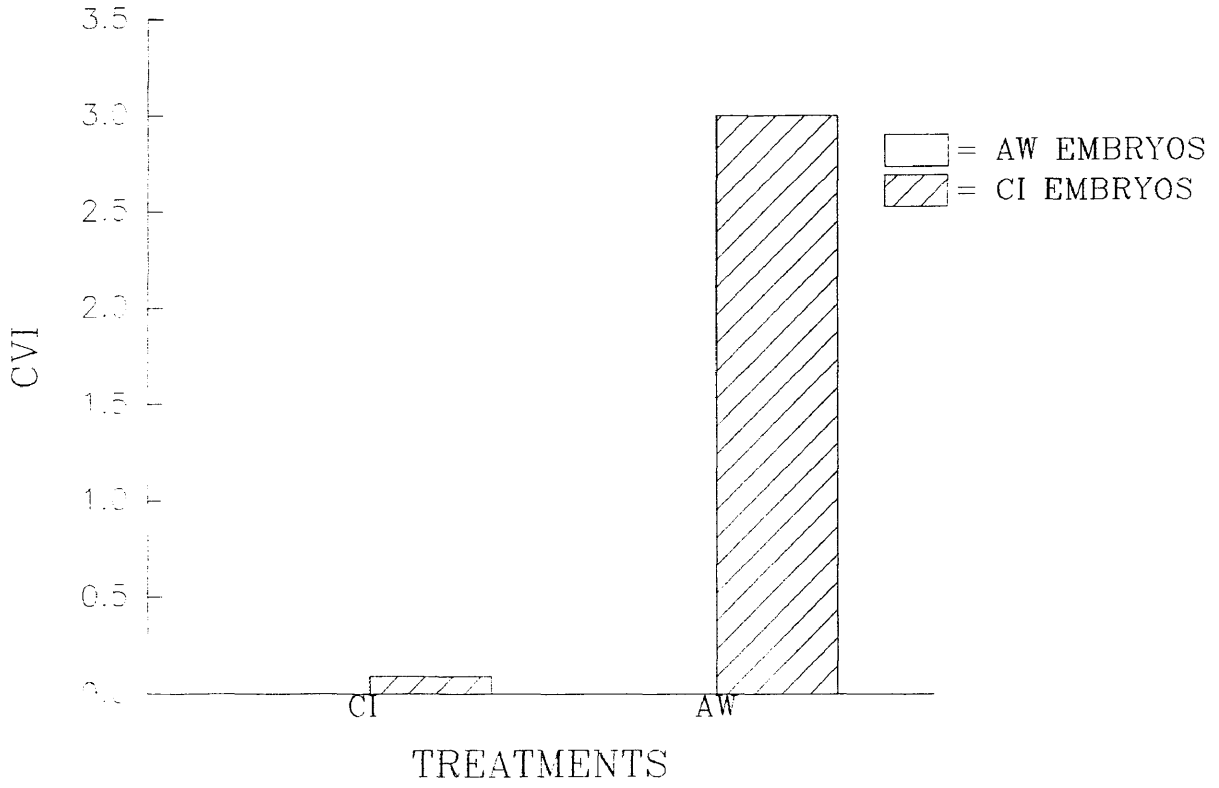
CARDIOVASCULAR ABNORMALITIES: DAY 10



limited by the number of mortalities which had occurred by that time. As on Day 5, no cardiovascular defects were observed in AW embryos in either treatment (average CVI=0). The CI embryos exposed to CI sediment again exhibited a low average CVI value (0.09), while those exposed to AW sediment exhibited a much higher average value (3.00). However, most of the CI embryos exposed to AW sediment were dead by Day 10, so the reported average CVI value was based on only 10-11 embryos per replicate. For this reason, statistical analyses were conducted using only Day 5 CVI data. Nevertheless, it is interesting to note that apparently the embryos with less severe defects on Day 5 (that caused the average CVI values to be less than 3.00) had worsened and/or died by Day 10, since only embryos with more severe defects were observed at that time (resulting in the average CVI value of 3.00).

AW and CI embryos responded similarly to CI sediment, with no instances of severe cardiovascular abnormalities being observed in either case. These results indicated that both groups of embryos were able to develop normally under the exposure conditions used in this experiment. However, chi-square analysis of the frequency of severely abnormal embryos in each AW sediment treatment (using pooled values from two replicates) showed a very significant difference between AW and CI embryos ($p<0.001$). The AW embryos were no more affected by AW sediment than by CI sediment, with no

severe abnormalities apparent in either treatment, while the CI embryos exhibited a significantly higher frequency of severe abnormalities in the AW sediment treatment than in the CI treatment ($p<0.001$).

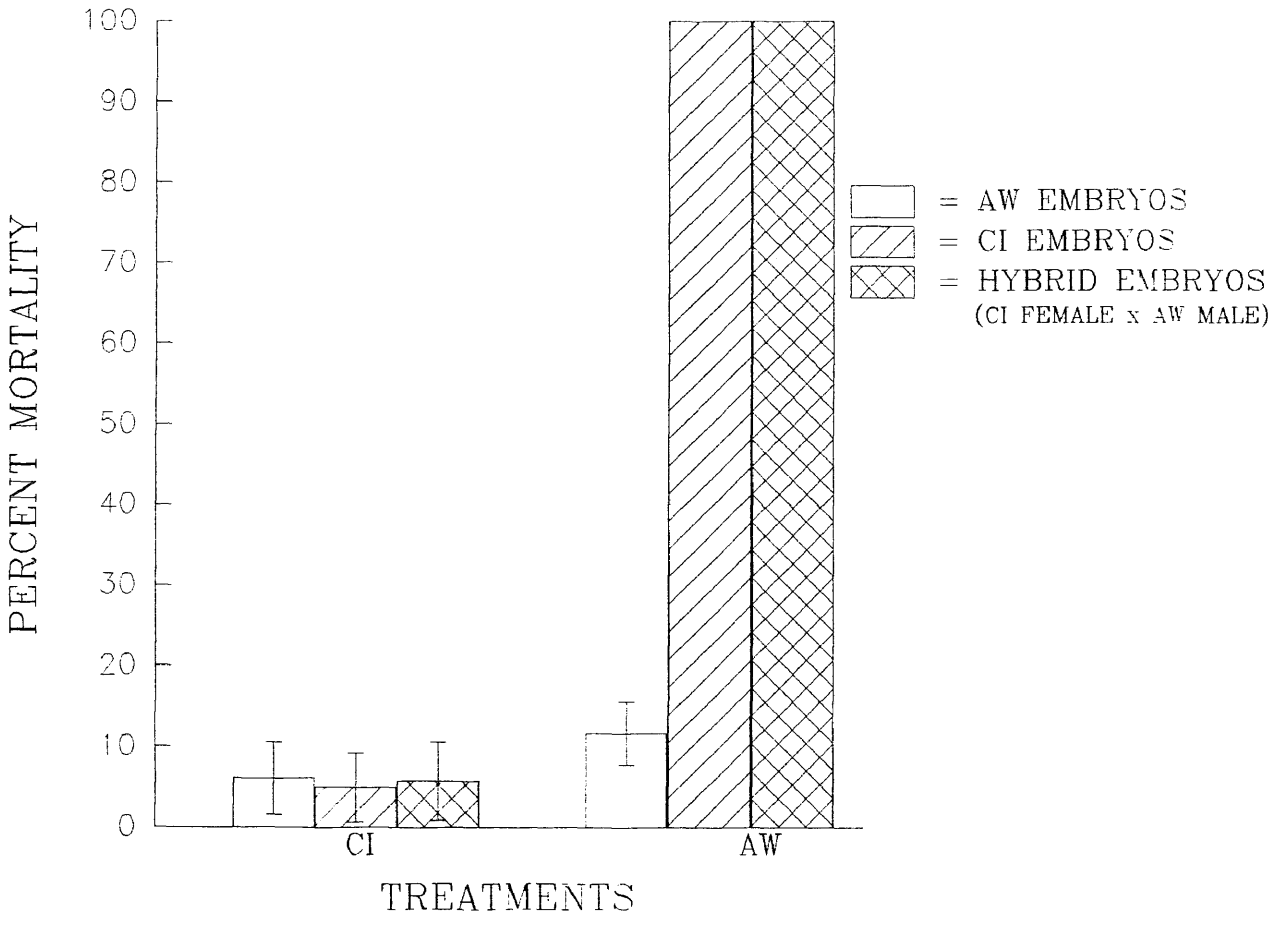
These results were very similar to those of Experiment I. No severe abnormalities were apparent in AW embryos exposed to the low dose of AW sediment in either experiment. At the same time, the CI embryos exposed to the contaminated sediment exhibited high frequencies of severe cardiovascular defects that were statistically similar in both experiments ($p=0.10$). However, the average Day 5 CVI value for these embryos was somewhat lower in the second experiment than in the first (2.72 versus 3.00), which suggested that the AW sediment used in Experiment II might not have been as teratogenic to susceptible embryos as that used in Experiment I or that the CI embryos somehow might have gained a degree of resistance. An alternative (and more likely) explanation is that the observed differences were due to random variations in sediment mixtures, embryo characteristics and experimental procedures.

Experiment III mortality The average mortality rates of AW, CI and hybrid (CI female x AW male) embryos exposed to AW and CI sediment are shown in Figure 10. As explained in the Methods section, each group consisted of separate batches of embryos from individual pairs of fish. This

Figure 10. Mean mortality rates for all treatments in Experiment III. The percent mortality in each replicate batch of embryos in a treatment was determined, and then these values were averaged to provide the mean mortality rate for that treatment. There were three replicates of each CI embryo treatment, four replicates of each AW embryo treatment and eight replicates of each hybrid embryo treatment. The vertical bars represent the standard deviations around the means (no variability was seen in the CI and hybrid embryo/AW sediment treatments).

EXPERIMENT III

MORTALITY



approach differed from the previous experiments, in which each treatment group consisted of replicates of embryos obtained from the pooled gametes of several fish. The overall viability of each batch of embryos was assessed by its response to CI sediment; if the mortality rate was greater than 15%, that batch was not included in the analysis of results. In this case, one of the batches of CI embryos was excluded for that reason (mortality on CI sediment = 20%).

As a group, CI embryos exhibited a nominal mortality rate when exposed to CI sediment (4.9%), but complete mortality when exposed to AW sediment (100%). In contrast, AW embryos exhibited relatively low mortality rates when exposed to either CI or AW sediment (6% and 11.6%, respectively). Hybrid embryos responded in a manner similar to CI embryos, with a nominal mortality rate on CI sediment (5.7%) and complete mortality on AW sediment (100%). The variability in response seen within each treatment group was important to consider in light of the use of separate batches of embryos as treatment replicates. In this case, no significant differences in mortality rates were observed within any of the treatments. This allowed data from all batches of embryos in a treatment to be pooled for statistical analysis.

Chi-square analysis of the frequency of mortality in each treatment showed that AW, CI and hybrid embryos

responded similarly to CI sediment ($p=0.95$). This result suggested that within the test parameters (i.e., considering only those embryo batches with 15% mortality or less), all three groups of embryos demonstrated similar abilities to survive under the exposure conditions used in this experiment.

As in the earlier experiments, however, clear differences were seen in the responses of AW and CI embryos to contaminated AW sediment. AW embryos exhibited a much lower mortality rate on AW sediment than did CI embryos; a difference which was shown to be very significant ($p<0.001$). The AW embryos were no more affected by AW sediment than by CI sediment ($p=0.097$), while the CI embryos were much more affected ($p<0.001$).

The hybrid embryos were found to be very similar in response to CI embryos. They exhibited the same high mortality rate (100%) when exposed to AW sediment as did the CI embryos; a rate which differed significantly from that seen when they were exposed to CI sediment ($p<0.001$). The hybrids exhibited a much higher mortality rate on AW sediment than did AW embryos; a difference which was found to be very significant ($p<0.001$).

Experiment III cardiovascular abnormalities The average CVI values of the groups of AW, CI and hybrid embryos exposed to AW and CI sediment are shown in Figure

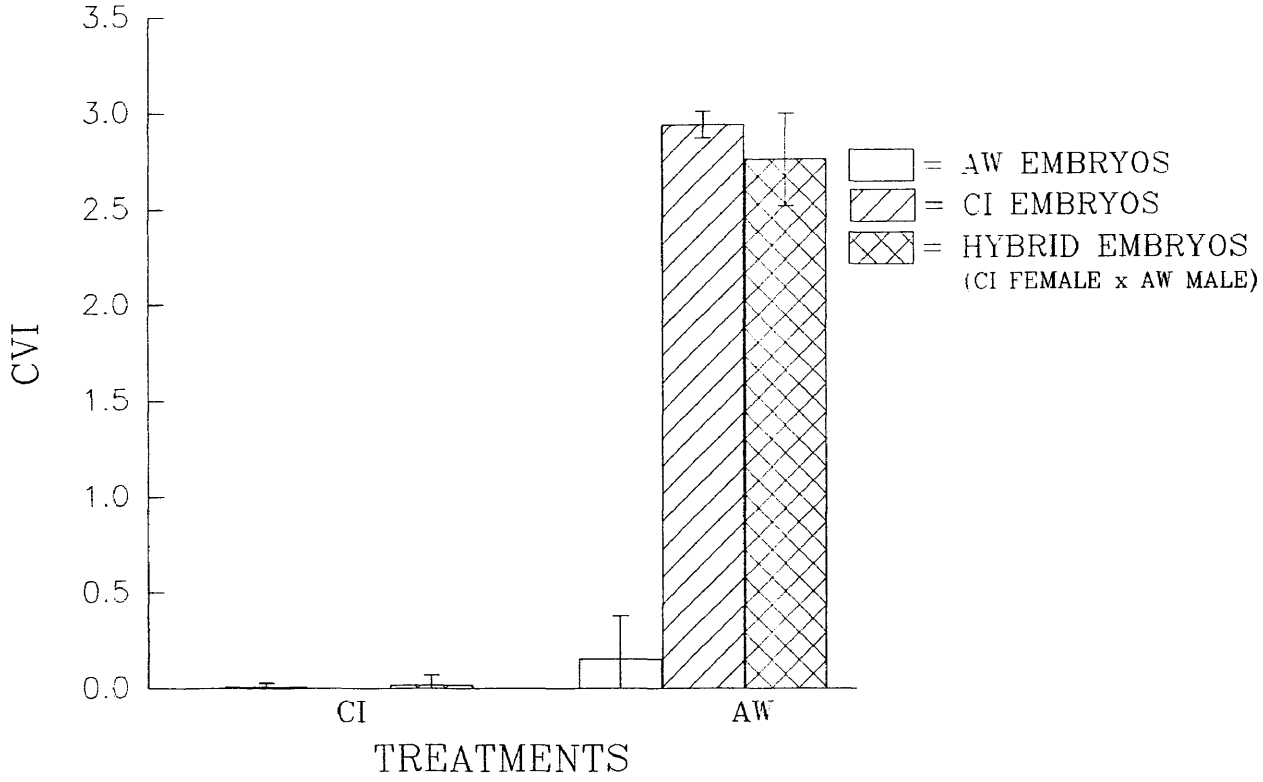
11. On Day 5, no cardiovascular abnormalities were apparent in CI embryos exposed to CI sediment (CVI=0), while many abnormalities were observed in CI embryos on AW sediment (average CVI=2.94). In contrast, AW embryos exhibited very low average CVI values on either CI or AW sediment (0.01 and 0.15, respectively). Hybrid embryos again responded in a manner similar to CI embryos, with a low average CVI value when exposed to CI sediment (0.02) and a much higher value on AW sediment (2.76).

The Day 10 CVI values also are shown in Figure 11, although as in the earlier experiments, their usefulness was limited because of high mortalities by this time. Again, AW embryos exhibited low average CVI values in both the CI and AW sediment treatments (0.03 and 0.40, respectively), although the value for the AW treatment was somewhat higher than on Day 5 (0.40 as compared to 0.15). As before, CI embryos exhibited a low average CVI value when exposed to CI sediment (0.04) and a much higher value on AW sediment (3.00). However, most of the CI embryos exposed to AW sediment had died by Day 10, so the reported treatment CVI value was based on only one batch of embryos. The hybrid embryo results were similar to those of Day 5, with a nominal average CVI value in the CI sediment treatment (0.02) and a much higher average value in the AW treatment (2.97). However, many of the hybrid embryos exposed to AW sediment had died by Day 10, so that only four replicates

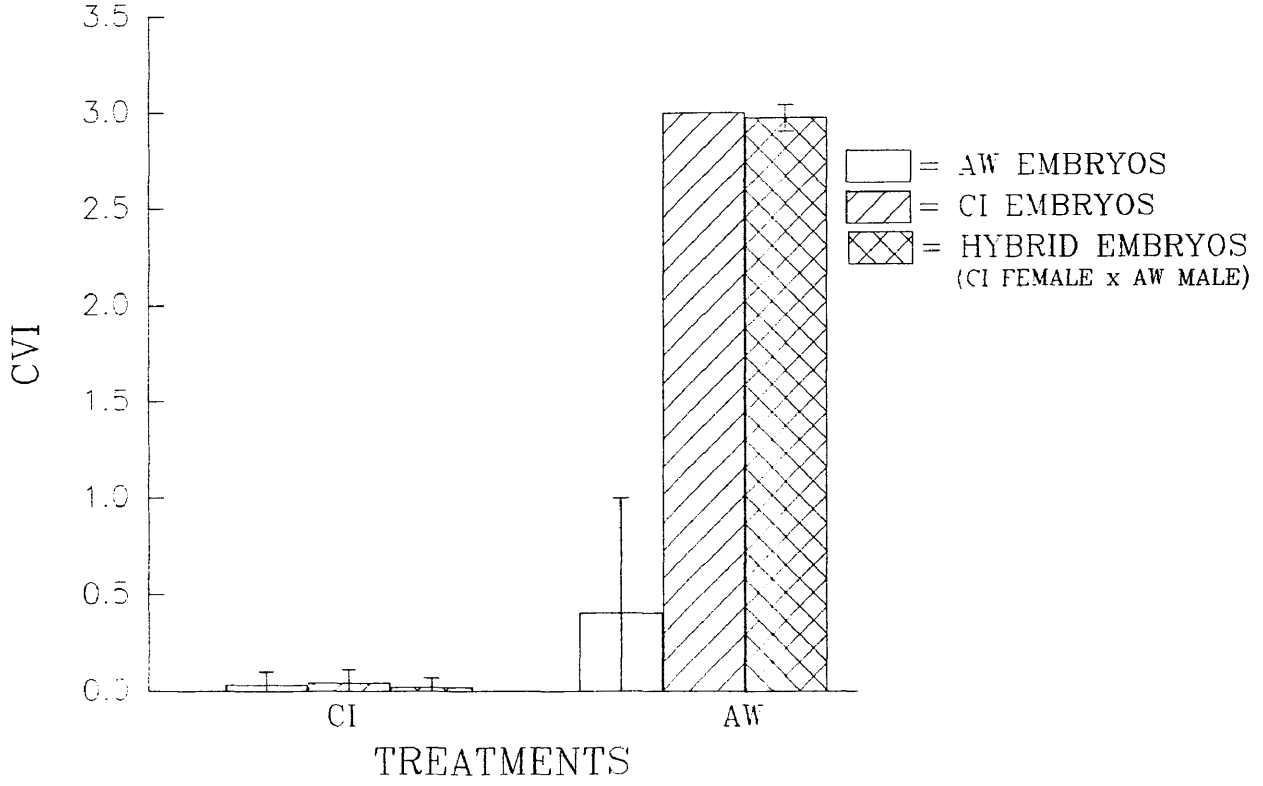
Figure 11. Mean cardiovascular index (CVI) values for all treatments in Experiment III. The mean CVI value for each replicate batch of embryos in a treatment was determined, and then these values were averaged to provide the overall mean CVI value for that treatment. There were three replicates of each CI embryo treatment, four replicates of each AW embryo treatment and eight replicates of each hybrid embryo treatment, except on Day 10, when there was only one replicate of the CI embryo/AW sediment treatment (all embryos in the other replicates were dead). The vertical bars represent the standard deviations around the means.

EXPERIMENT III

CARDIOVASCULAR ABNORMALITIES: DAY 5



CARDIOVASCULAR ABNORMALITIES: DAY 10



contained more than 10 embryos.

On both days, some of the treatment groups exhibited more variability in the frequencies of severe abnormalities than had been seen with mortality rates. The average CVI value of each batch of embryos exposed to AW sediment is shown in Figure 12. This graph illustrates the degree of variability found both within treatments and within batches, particularly among the AW and hybrid embryos. Chi-square analysis of the frequency of severe cardiovascular defects in each batch of embryos found significant differences among replicates of the AW embryo/AW sediment treatment on both Day 5 and Day 10, as well as among replicates of the hybrid embryo/AW sediment treatment on Day 5.

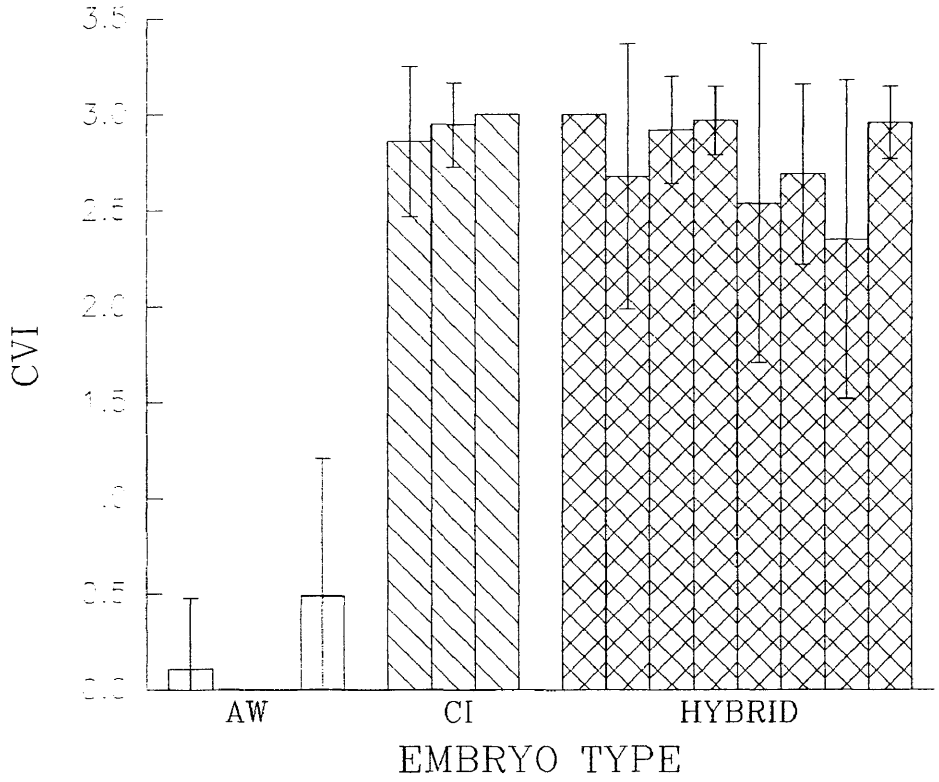
Statistical analysis of the differences in response among the various treatments was limited by the significant variability in some of the treatments, which prevented the pooling of data from all replicates. Thus, in many cases, treatment results were compared qualitatively (using average CVI values) rather than analyzed statistically. Day 10 results were used for some of the treatment comparisons, when it was felt that the number of mortalities did not compromise the validity of the data.

All three groups of embryos were found to react similarly to CI sediment on Day 5 (with no severe abnormalities observed in any group) and on Day 10, when chi-square analysis of the frequency of severe abnormalities

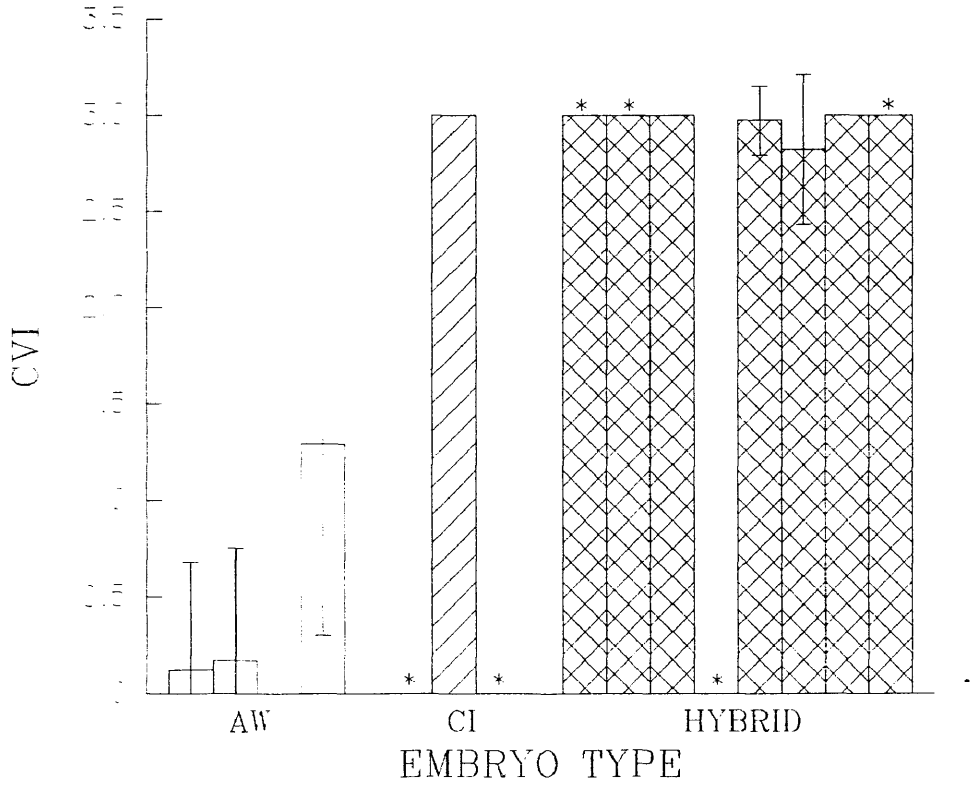
Figure 12. Mean cardiovascular index (CVI) values for each batch of embryos exposed to AW sediment in Experiment III. The mean CVI value for each batch was determined by averaging the individual CVI values for each embryo in the batch. The vertical bars represent the standard deviations around the means (no variability was seen in some of the CI and hybrid embryo batches, especially on Day 10). The asterisks denote batches containing less than 10 embryos (due to mortality).

EXPERIMENT III

BATCH CVI VALUES: DAY 5



BATCH CVI VALUES: DAY 10



in each group found no significant differences ($p=0.57$). These results suggested that within the test parameters, the three groups of embryos displayed no inherent differences in their ability to develop normally under the exposure conditions used in this study.

As in the earlier experiments, however, AW and CI embryos exhibited clear differences in their ability to develop normally when exposed to AW sediment. On Day 5 (Day 10 results were not compared due to the lack of replicates in the CI group), AW embryos exhibited a much lower average CVI value on AW sediment than did CI embryos (0.15 versus 2.94). A comparison of average CVI values indicated that AW embryos were not much more affected by AW sediment than by CI sediment, while chi-square analysis showed that the CI embryos were significantly more affected ($p<0.001$).

The hybrid embryos again appeared to be most similar in response to the CI embryos. On Day 5 (as above, Day 10 CI embryo results were not used), hybrid embryos exposed to AW sediment exhibited an average CVI value only slightly lower than that of exposed CI embryos (2.76 versus 2.94). This value was much higher than that seen when the hybrid embryos were exposed to CI sediment (2.76 versus 0.02). In addition, on both Day 5 and Day 10 the hybrid embryos exhibited a much higher average CVI value when exposed to AW sediment than did AW embryos (Day 5: 2.76 versus 0.15; and Day 10: 2.97 versus 0.40).

Experiment IV mortality The average mortality rates of AW, CI and hybrid (AW female x CI male) embryos exposed to AW and CI sediment are shown in Figure 13. As in Experiment III, each group consisted of separate batches of embryos from individual pairs of fish. In this case, all batches of embryos had less than 15% mortality when exposed to CI sediment, so all batches were included in the analysis of results.

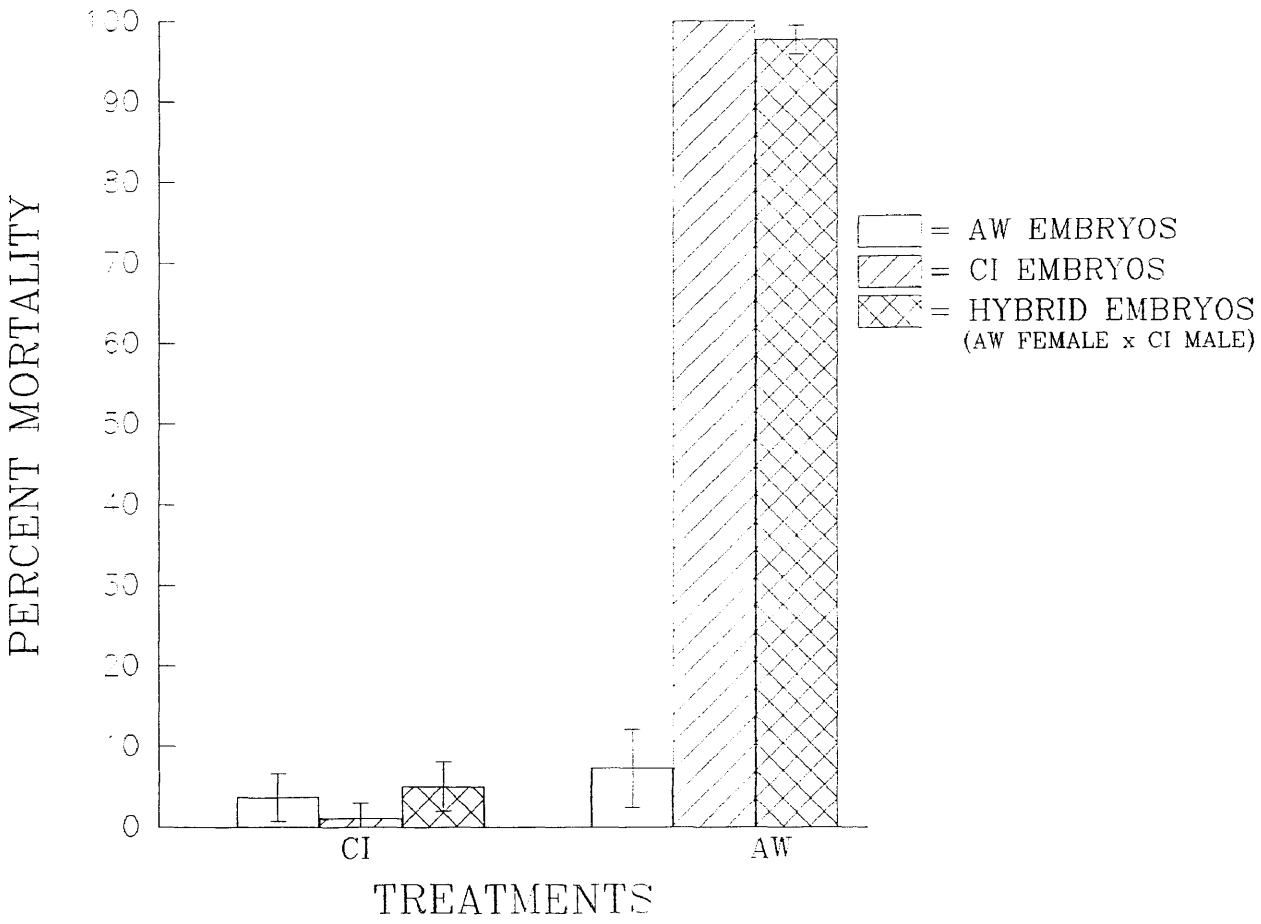
As before, the CI embryos exhibited a nominal average mortality rate (1.0%) when exposed to CI sediment, but complete mortality (100%) when exposed to AW sediment. In contrast, AW embryos exhibited relatively low mortality rates when exposed to either CI or AW sediment (3.7% and 7.3%, respectively). The hybrid embryos again responded in a manner similar (but not identical) to CI embryos, with a nominal mortality rate on CI sediment (5.0%) and a much higher rate on AW sediment (97.7%).

As in Experiment III, the use of separate batches of embryos as treatment replicates made the variability in response within each treatment group an important factor to be considered in the analysis of results. In this case, no statistically significant differences in mortality rates were observed within any of the treatments, although the batches of hybrid embryos exposed to AW sediment did show some variability that was close to being significant ($p=0.102$). This allowed data from all batches of embryos in

Figure 13. Mean mortality rates for all treatments in Experiment IV. The percent mortality in each replicate batch of embryos in a treatment was determined, and then these values were averaged to provide the mean mortality rate for that treatment. There were four replicates of each CI and AW embryo treatment and eight replicates of each hybrid embryo treatment. The vertical bars represent the standard deviations around the means (no variability was seen in the CI embryo/AW sediment treatment).

EXPERIMENT IV

MORTALITY



a treatment to be pooled for statistical analysis.

Chi-square analysis of the frequency of mortality in each treatment showed that AW, CI and hybrid embryos responded similarly to CI sediment ($p=0.14$). This result suggested that all three groups of embryos were able to survive equally well under the exposure conditions used in this experiment.

As in all previous experiments, however, AW and CI embryos exhibited major differences in their responses to contaminated AW sediment. AW embryos exhibited a much lower mortality rate on AW sediment than did CI embryos; a difference which was shown to be very significant by chi-square analysis ($p<0.001$). The AW embryos were no more affected by AW sediment than by CI sediment ($p=0.19$), while the CI embryos were much more affected ($p<0.001$).

The hybrid embryos were found to be most similar in response to the CI embryos, although some differences were apparent. The hybrids exhibited a high mortality rate when exposed to AW sediment, as did the CI embryos; a rate which differed significantly from that seen when they were exposed to CI sediment ($p<0.001$). However, this rate was somewhat lower than that seen in CI embryos exposed to AW sediment; a difference which was found to be significant ($p=0.04$). Nevertheless, the hybrids did not appear to be very similar to AW embryos. They exhibited a much higher mortality rate on AW sediment than did AW embryos; a difference that was

shown to be very significant ($p<0.001$).

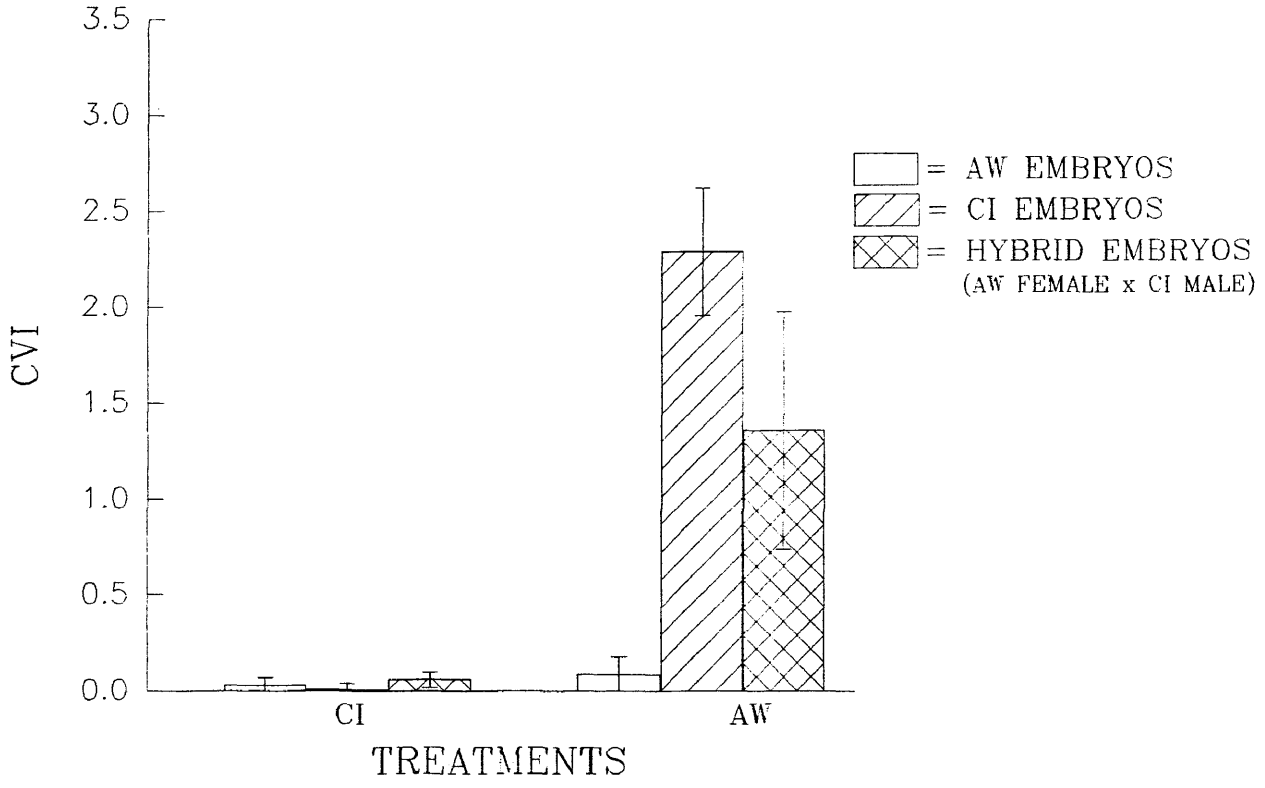
Experiment IV cardiovascular abnormalities The average CVI values of the groups of AW, CI and hybrid embryos exposed to AW and CI sediment are shown in Figure 14. On Day 5, scarcely any cardiovascular abnormalities were apparent in CI embryos exposed to CI sediment (average CVI=0.01), while many abnormalities were observed in CI embryos on AW sediment (average CVI=2.29). In contrast, AW embryos exhibited very low average CVI values on either CI or AW sediment (0.03 and 0.09, respectively). Hybrid embryos exhibited a response intermediate to that seen with AW or CI embryos, with a low average CVI value when exposed to CI sediment (0.06) and a moderate average value (1.36) on AW sediment.

The Day 10 CVI values (also shown in the figure) were more useful than in earlier experiments, due to the limited number of mortalities which had occurred by this time. Again, AW embryos exhibited low average CVI values in both the CI or AW sediment treatments (0.07 and 0.12, respectively). As before, CI embryos exhibited a low average CVI value when exposed to CI sediment (0.07) and a much higher value on AW sediment (2.98). The hybrid embryo results differed somewhat from Day 5, becoming more similar to those seen with CI embryos. They exhibited a low average CVI value in the CI sediment treatment (0.13), and a much

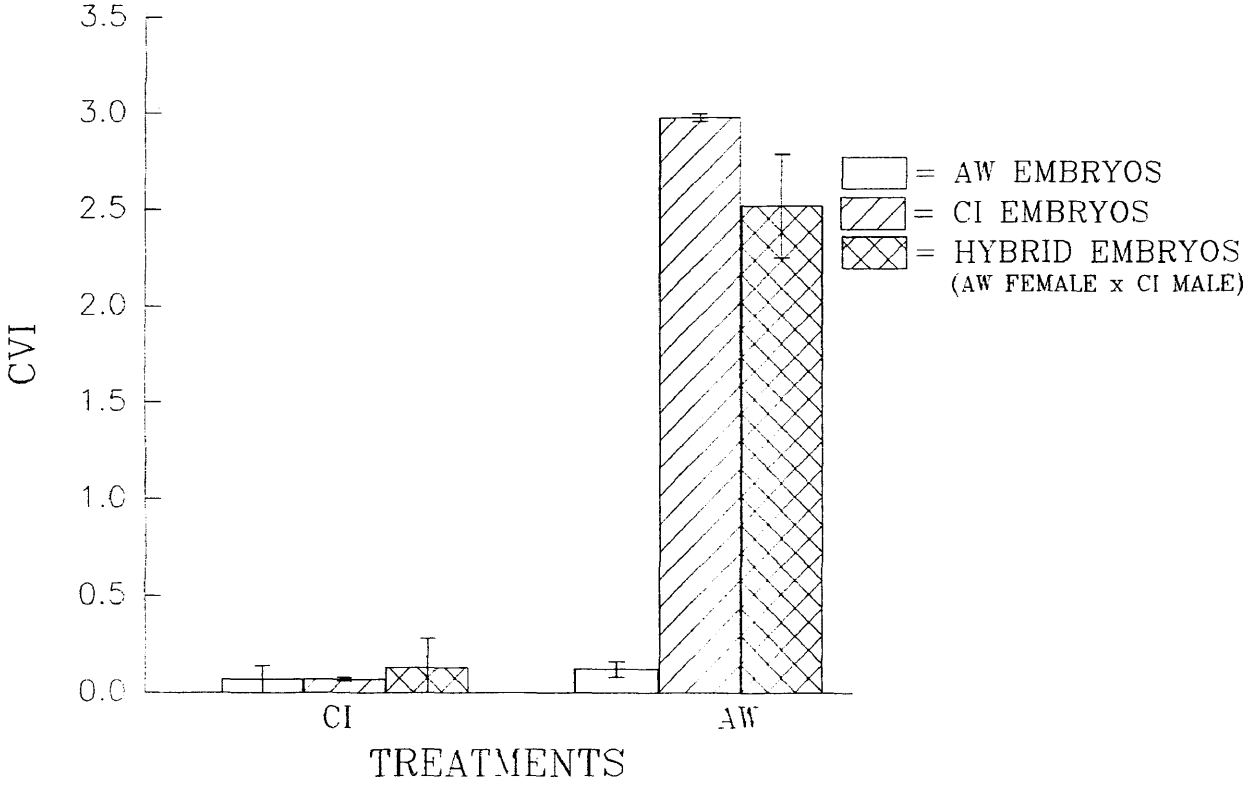
Figure 14. Mean cardiovascular index (CVI) values for all treatments in Experiment IV. The mean CVI value for each replicate batch of embryos in a treatment was determined, and then these values were averaged to provide the overall mean CVI value for that treatment. There were four replicates of each CI and AW embryo treatment and eight replicates of each hybrid embryo treatment. The vertical bars represent the standard deviations around the means.

EXPERIMENT IV

CARDIOVASCULAR ABNORMALITIES: DAY 5



CARDIOVASCULAR ABNORMALITIES: DAY 10



higher average value in the AW treatment (2.52).

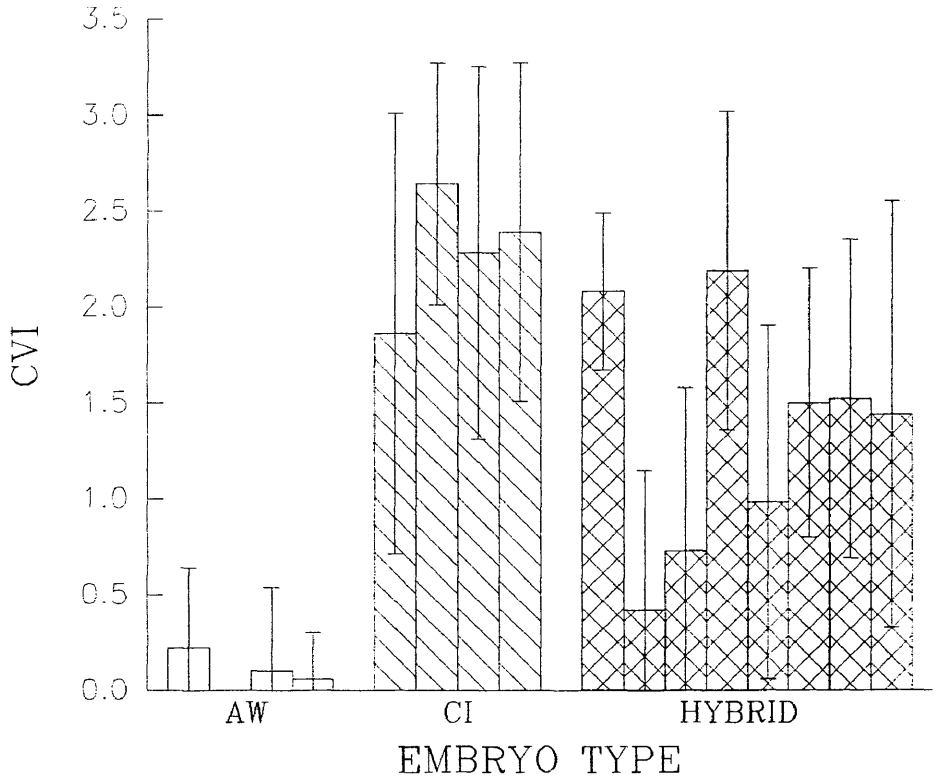
As in Experiment III, some of the treatment groups exhibited more variability in the frequencies of severe abnormalities than had been seen with mortality rates. The average CVI value of each batch of embryos exposed to AW sediment is shown in Figure 15. This graph illustrates the degree of variability found both within treatments and within batches, particularly among the CI and hybrid embryos. Chi-square analysis of the frequency of severe cardiovascular defects in each batch of embryos found significant differences among replicates of the CI embryo/AW sediment treatment on Day 5, the hybrid embryo/CI sediment treatment on Day 10, and the hybrid embryo/AW sediment treatment on Day 5.

It was interesting to note that while replicates of the hybrid embryo/AW sediment treatment on Day 10 appeared to vary substantially in terms of their average CVI values, no significant differences were observed in their frequencies of severe abnormalities. The lack of variability in the latter case apparently was caused by the loss of discrimination that occurred when three types of cardiovascular defects (CVI values=2-4) were combined into one "severe abnormalities" category. Thus, the interpretation of experimental results would have differed depending on which parameter was used to measure treatment effects; the average CVI value or the frequency of severe

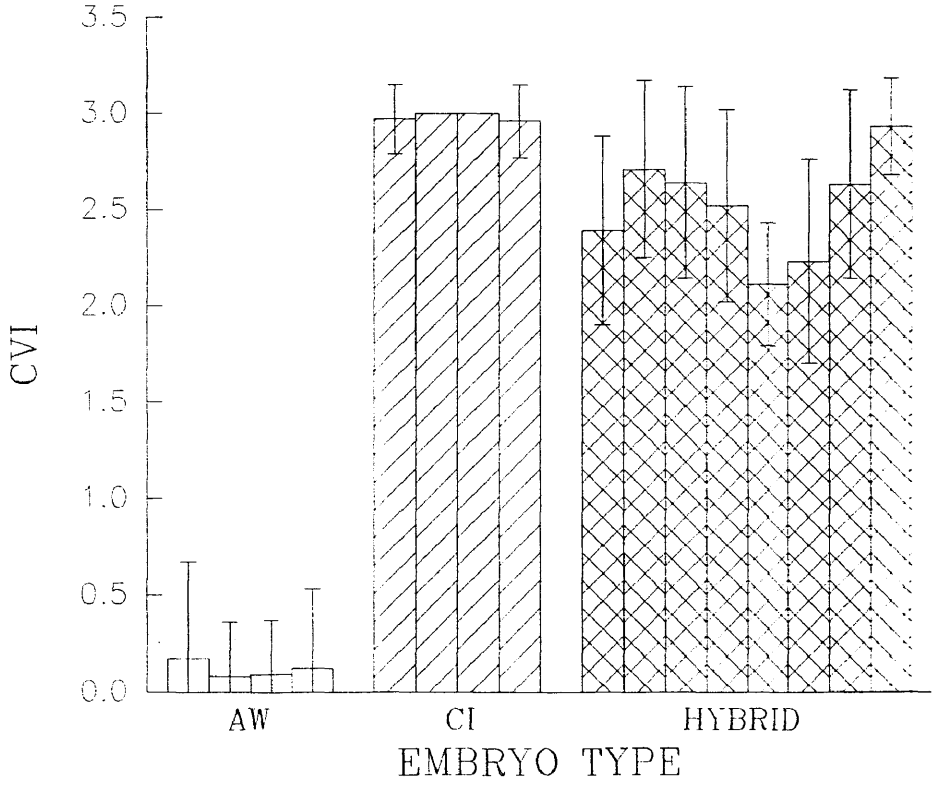
Figure 15. Mean cardiovascular index (CVI) values for each batch of embryos exposed to AW sediment in Experiment IV. The mean CVI value for each batch was determined by averaging the individual CVI values for each embryo in the batch. The vertical bars represent the standard deviations around the means (no variability was seen in two of the CI embryo batches on Day 10).

EXPERIMENT IV

BATCH CVI VALUES: DAY 5



BATCH CVI VALUES: DAY 10



abnormalities. While either interpretation would have been valid, statistical analysis was possible only with the combined severe abnormalities data (as explained in the Methods section). Therefore, statistical analyses and interpretations of this experiment and all previous experiments were based primarily on results obtained using the frequency of severe abnormalities as a measure of treatment effects.

As in Experiment III, statistical analysis of the differences in response among the various treatments was limited by the significant variability in some of the treatments, which prevented the pooling of data from all replicates. Thus, in many cases, treatment results were compared qualitatively (using average CVI values) rather than analyzed statistically. Results from both Day 5 and Day 10 were used for the treatment comparisons, as the limited number of mortalities that had occurred by Day 10 should not have compromised the validity of those data.

All three groups of embryos were found to respond similarly to CI sediment on Day 5, when chi-square analysis of the frequency of severe abnormalities in each group found no significant differences ($p=0.13$). On Day 10, only the AW and CI embryos could be compared statistically and again, they demonstrated no significant differences in response ($p=1.00$). By inspection of the data, it appeared that the hybrid embryos also responded in a similar fashion. These

results suggested that the three groups of embryos were able to develop equally well under the exposure conditions used in this experiment, although the hybrid embryos exhibited somewhat more variability in this regard than did the other embryos.

As in all previous experiments, however, AW and CI embryos exhibited clear differences in their responses to contaminated AW sediment. On Day 5, AW embryos exhibited a much lower average CVI value on AW sediment than did CI embryos (0.09 versus 2.29). Chi-square analysis showed that AW embryos were not affected significantly more by AW sediment than they were by CI sediment ($p=1.00$), while a comparison of average CVI values indicated that CI embryos were much more affected by AW sediment. Similar results were noted on Day 10, when chi-square analysis found a very significant difference between the responses of AW and CI embryos to AW sediment ($p<0.001$). Again, the AW embryos responded similarly to both AW and CI sediment in terms of the frequency of severe defects ($p=0.26$), while CI embryos responded very differently in the two treatments ($p<0.001$).

The hybrid embryos appeared to be similar in response to CI embryos, although the similarity was not as strong as that seen in Experiment III. On Day 5, hybrid embryos exposed to AW sediment exhibited an average CVI value (1.36) intermediate between that seen with exposed CI (2.29) or AW embryos (0.09). However, the hybrids resembled CI embryos

much more than AW embryos in showing a higher average CVI value on AW sediment than on CI sediment (1.36 versus 0.06).

The hybrid resemblance to CI embryos was stronger by Day 10. Chi-square analysis found no significant differences in the frequency of severe abnormalities in these groups of embryos when exposed to AW sediment ($p=0.99$), although the two groups did appear to differ somewhat in terms of average CVI values. Again, the hybrid embryos exhibited a much higher average CVI value on AW sediment than on CI sediment (2.52 versus 0.13), as did the CI embryos. The hybrids did not appear to be similar to the AW embryos. They exhibited a much greater frequency of severe abnormalities on AW sediment than did AW embryos; a difference that was shown to be very significant ($p<0.001$).

DISCUSSION

Toxicity Resistance in Mummichog from a Contaminated Site

The results of this study support the hypothesis that mummichog from the chemically contaminated Atlantic Wood (AW) site have developed a resistance to the acute toxicity of the pollutants in their environment. This resistance is not shared by mummichog from the Catlett Island (CI) reference site. In all experiments, AW mummichog embryos exposed to AW sediment developed and hatched relatively normally, while similarly exposed CI embryos developed cardiovascular abnormalities and died. The differences in response between embryos from the two populations were extremely significant, using either cardiovascular defects or mortality as an end point.

Resistance to environmental pollutants has been documented in several fish populations, as described in the Introduction. Klerks and Weis (1987) included many of these studies in their review of the literature on resistance to heavy metals in aquatic organisms, and Mulvey and Diamond (1991) provided an additional summary of representative studies from this literature. Other researchers have

reported increased resistance in fish populations exposed to organic pollutants, such as phenol and insecticides (Vinson et al. 1963; Culley and Ferguson 1969; Norup 1972; Fabacher and Chambers 1973; Angus 1983; Andreasen 1985).

In contrast, certain studies have shown that some fish populations inhabiting contaminated environments do not adapt to the chemical pollution. For example, Bishop and McIntosh (1981) found that bluegill (*Lepomis macrochirus*) from a metal-contaminated lake exhibited the same level of cadmium tolerance as did fish from an uncontaminated lake. Rahel (1981) found that common shiners (*Notropis cornutus*) from a zinc-polluted stream were no more zinc tolerant than were shiners from nearby unpolluted streams. In addition, multiple generations of selection for zinc tolerance in a laboratory population of flagfish (*Jordanella floridae*) failed to result in increased tolerance in these fish. In their review, Klerks and Weis (1987) noted that while such reports are not as common as are reports documenting increased resistance, this could be due to a general lack of interest in negative results. They suggested that the results of selection experiments and the often observed reductions in species diversity in metal-polluted environments support the hypothesis that aquatic species differ in their ability to adapt to these environments.

Despite the relatively abundant literature on fish resistance to environmental pollutants (especially to heavy

metals and pesticides), there have been no studies of fish populations demonstrating resistance to polycyclic aromatic hydrocarbons (PAH). This lack of information is unfortunate, because PAH appear to be the dominant contaminants at many industrialized sites, including the AW site. Extremely high concentrations of PAH have been identified in the sediments at this location (Vogelbein et al. 1990; Buchman et al. 1992), presumably due to spills or leakage of creosote from the Atlantic Wood facility. PAH can be acutely toxic to aquatic organisms, and the metabolic intermediates of some PAH have been shown to be mutagenic, carcinogenic and/or teratogenic (Rand and Petrocelli 1985). Several field and laboratory studies have documented adverse effects ranging from immunological changes to mortality in fishes exposed to PAH-laden sediments from the Elizabeth River (Hargis et al. 1984; Weeks and Warinner 1984; Weeks et al. 1986; Huggett et al. 1987; Roberts et al. 1989; Vogelbein et al. 1990).

In Experiment I of this study, creosote-amended sediment was tested as a possible toxic substitute for AW sediment. The results of this experiment were equivocal. CI embryos appeared to be adversely affected by the toxicity of this sediment in a manner similar to that seen with AW sediment, but not to the same degree. The discrepancy could have been due to the reduced amount of PAH in the creosote-amended sediment as compared to even the lowest AW sediment

treatment. However, due to analytical difficulties (as explained in the Results under Chemical Analyses), this measurement was not conclusive. The discrepancy might have been due instead to the lack of some key toxicant (other than PAH) in the creosote-amended sediment that is present in AW sediment. Some of the contaminants that have been identified at the AW site include pentachlorophenol (PCP), polychlorinated dibenzo-p-dioxins (PCDD), polychlorinated dibenzofurans (PCDF), arsenic, copper and zinc (Buchman et al., 1992).

In either case, further studies with greater amounts of creosote and better quality control are required before the feasibility of substituting creosote for the toxicants in AW sediment can be evaluated properly. Additional experiments testing the responses of AW and CI embryos to specific PAH known to be present in AW sediment (singly and in combination) could provide the necessary evidence to identify PAH as the effective contaminants at the AW site. For the present study, however, PAH concentration was used only as an index of the contamination level of each sediment treatment. These experiments were designed to examine differences in resistance to contaminated sediment among groups of embryos; determination of the mechanism(s) of toxicity was left for future studies.

Cardiovascular Defects in Susceptible Embryos

In all experiments of this study, susceptible embryos exposed to contaminated sediment exhibited varying degrees of abnormal cardiovascular development. These terata are similar to those described in many other studies, as reviewed by Weis and Weis (1989b). For example, Weis and Weis (1977a,b) observed tube hearts, reduced circulation and pericardial swelling in mummichog embryos exposed to mercury or methylmercury; Middaugh et al. (1988) observed tube hearts and reduced circulation in embryos of the inland silverside (*Menidia beryllina*) exposed to 2,4-dinitrophenol, naphthalene or "produced water" (a by-product of oil and gas production; and Marty et al. (1990) observed pericardial edema and heart malformations in Japanese medaka (*Oryzias latipes*) embryos exposed to N-nitroso compounds. The authors of the latter study made a clear distinction between true tube hearts, which are primary cardiac malformations, and "stretched" hearts, which are secondary cardiac malformations that develop as a result of pericardial edema. By these criteria, the severe tube hearts observed in the present study are true tube hearts, while the less severe heart defects are "stretched" hearts.

It is apparent from these studies that cardiovascular defects are fairly nonspecific terata that can be caused by a number of chemicals in diverse fish species.

Nevertheless, they can be extremely useful as end points in toxicity tests. Cardiovascular abnormalities meet the requirements for good end points of toxicity put forward by Rand and Petrocelli (1985) in that they are "unequivocal, clearly relevant, readily observable, describable, measurable, biologically significant, and reproducible." In addition, these terata often become evident fairly early in development, before total mortality or successful hatch can be assayed, which could reduce the time required to complete a toxicity test.

In the present study, both cardiovascular malformations and mortality were used as toxicity end points. These effects appeared to occur as a timeline of events, with cardiovascular defects usually leading to death. Thus, in most cases, interpretation of experimental results was the same regardless of whether cardiovascular defects or mortality was used as an end point. There were some important exceptions, however. In the first experiment, AW embryos exposed to the medium and high doses of AW sediment exhibited significantly more mortality than embryos exposed to the other sediment treatments, whereas no significant differences among treatments were noted in terms of cardiovascular defects. Throughout the study, variability in mortality was noted only among replicates of the CI embryo/creosote treatment, while variability in cardiovascular abnormalities was observed among replicates

of several treatments. In addition, the similarity of the hybrid embryos to CI embryos in the last two experiments usually was stronger when mortality was used as an end point than when cardiovascular malformation was used.

One possible explanation for the contrasting results is that the classification of cardiovascular defects is strongly dependent on embryo age at the time of screening. While embryos were screened on the same days in each experiment, variations in rates of development among individuals and across experiments could have affected the severity of the defects observed at these times. However, since total mortality was not scored until the end of each experiment, it was not affected by the rate of development of the embryos. The feasibility of this explanation is supported by the fact that differences between the two end points were less apparent when the results of the Day 10 cardiovascular screening were considered instead of the Day 5 results.

Another possible explanation for the observed differences between the two end points is that mortality encompasses a wider range of effects than does cardiovascular malformation. In this study, mortality included dead embryos, unhatched (deformed) embryos, severely deformed larvae and dead larvae. Mortality was not preceded by cardiovascular malformation in every case. In a sense, the two end points measure different aspects of

sediment toxicity, and thus some discrepancies in the interpretation of experimental results are to be expected.

Genetic Adaptation versus Physiological Acclimation

The results of this study support the hypothesis that the toxicity resistance observed in AW mummichog is due to genetic adaptation rather than physiological acclimation. In all experiments, the AW mummichog embryos that demonstrated an increased resistance to the toxicity of contaminated sediment had never before been in direct contact with any contaminants. Thus, the resistance seen in these embryos should not have been due to acclimation to the sediment toxicants, but rather to a heritable adaptation.

It is possible, however, that the embryos had become acclimated to AW toxicants indirectly while the eggs were developing in the female fish. Weis and Weis (1989b) noted that one of the ways embryos can be exposed to pollutants is via toxicant incorporation into the egg yolk during oogenesis in exposed females. They summarized a few studies that demonstrated that malformations can occur in embryos developing from eggs taken from toxicant-exposed females (e.g., Smith and Cole 1973; Speranza et al. 1977; Birge et al. 1979). The potential for acclimation due to this type of indirect exposure was minimized in the early experiments

by maintaining fish in clean water for several weeks before stripping them of eggs. However, oogenesis might have occurred prior to this time while the fish were still at the contaminated site, and the maintenance period in clean water might not have been sufficient for the fish and eggs to depurate.

The results of Experiment II contradict this indirect acclimation hypothesis and provide further support of the theory that toxicity resistance in AW mummichog is due to genetic adaptation. In this experiment, AW embryos obtained from the same female fish used in Experiment I were much more resistant to the teratogenic and lethal effects of contaminated sediment than were CI embryos. The similarity of these results to those of Experiment I suggested that the second batch of AW embryos was no less resistant than the first batch of embryos, despite the seven weeks the parental fish had spent in clean water between these experiments. In contrast, if the resistance had been due to indirect acclimation, the second batch of embryos would have been expected to be less resistant than the first batch.

Additional evidence in support of the adaptation hypothesis is provided by the results of the final experiment. The AW female x CI male hybrids did not exhibit resistance similar to AW purebreds, as would be expected if the resistance was due solely to indirect acclimation of embryos via toxicants stored in eggs during oogenesis.

Rather, the hybrids appeared to be more similar to CI embryos in terms of their resistance responses. These results indicate that the male plays a role in determining the resistance of the offspring, and thus genetic factors must be involved in toxicity resistance.

Several of the published studies on toxicity resistance in fish populations attributed the observed tolerance to genetic adaptation. For example, mosquitofish (*Gambusia affinis*) resistance to both phenol and insecticides was found to be due at least in part to genetic adaptation (Fabacher and Chambers 1973; Angus 1983; Andreasen 1985). Methylmercury tolerance in mummichog embryos from a polluted environment was postulated to have a genetic basis, given that tolerance was associated with the fin ray count of the female (Weis et al. 1982) and no evidence could be found for physiological acclimation (Weis et al. 1985).

As Weis et al. (1981a; 1985) noted, the mummichog has a high probability of being able to adapt to pollutant stress because of its genetic plasticity. Luoma (1977) observed that resistance usually occurs in opportunist species which have a large variety of genotypes upon which selection can act. The mummichog has been shown to be among the most genetically variable of all teleost species that have been examined (Smith and Fujio 1982). In addition, the polygynous mating system of the mummichog is thought to allow for rapid evolutionary response to changes in the

environment (Mitton and Koehn 1975).

Inheritance of the Resistance Trait

The results of the final two experiments are consistent with the hypothesis that mummichog resistance to the toxicity of contaminated sediment is inherited as a recessive trait that can be modified by maternal effects. The evidence also suggests that this trait is likely to be autosomal rather than sex-linked.

In Experiment III, hybrid embryos were obtained by crossing susceptible CI females with resistant AW males. These embryos responded to AW sediment in a manner very similar to CI embryos; with complete mortality and a high prevalence of severe abnormalities. One possible explanation for these results is that the resistance trait is genetically determined and susceptibility to the toxic effects of the AW sediment is dominant over resistance. Another possibility is that the level of offspring resistance is determined mainly by the level of maternal resistance, due to some type of non-genetic effect.

The results of Experiment IV provide some of the information necessary to evaluate these two hypotheses. In this experiment, hybrid embryos were obtained by crossing resistant AW females with susceptible CI males. The results obtained using mortality as an end point were very similar

to those seen in Experiment III. In both cases, CI embryos exhibited 100% mortality when exposed to AW sediment, while AW embryos on AW sediment exhibited nominal mortality rates that did not differ significantly between experiments ($p=0.16$). As explained in the Methods section, the purebred embryos were used as benchmarks of the degree of susceptibility or resistance in each parental strain under the specific exposure conditions used in each experiment. The fact that they responded in the same way in Experiments III and IV made it possible to compare directly the responses of the hybrid embryos in both experiments. The average mortality rate of hybrid embryos exposed to AW sediment was slightly lower in Experiment IV than in Experiment III; a difference that was found to be statistically significant ($p=0.02$).

The fact that the hybrids in both Experiments III and IV responded similarly to CI embryos supports the hypothesis that the resistance trait is genetically determined and susceptibility to the lethal effects of AW sediment is dominant over resistance. However, the significantly lower mortality rate observed in hybrids in Experiment IV as compared to Experiment III suggests that something other than genetic factors might be involved in determining hybrid response. Since these hybrids differ from those in Experiment III only in the sex of the resistant parent (in this case, the female), it appears that non-genetic maternal

effects could be involved in moderating the expression of the susceptibility phenotype.

Further support for this hypothesis is provided by analysis of the results of Experiment IV using cardiovascular malformation as an end point. Once again, these results were analogous to those seen in Experiment III, with a few notable differences. In both studies, AW embryos exposed to AW sediment exhibited nominal average CVI values, while CI embryos on AW sediment exhibited much higher average CVI values. Statistical comparisons between experiments were not possible due to significant variability in several of the treatments. However, the average CVI value of the CI embryo/AW sediment treatment on Day 5 was appreciably lower in Experiment IV than in Experiment III. In addition, the average CVI value of the AW embryo/AW sediment treatment on Day 10 was slightly lower in Experiment IV than in Experiment III.

There are several factors which could have been responsible for the relatively lower average CVI values observed in Experiment IV (as well as for the lower mortality observed by Day 10). Some of the batches of embryos obtained for this experiment (both hybrids and purebreds) exhibited a slower rate of development than had been seen in batches from the previous study, perhaps related to crowding in the exposure bowls due to the larger batch size. The slower development meant that many of these

embryos might not have had time to form severe abnormalities by Day 5 or even by Day 10. Alternatively, the stock sediment may have lost some of its teratogenicity. This does not seem very likely, however, as Experiment IV began only two weeks after Experiment III. Finally, random variation in experimental procedure and sediment mixtures and among individual pairs of fish also could have affected the results of each experiment.

Regardless of the reason(s), the differential responses exhibited by the purebred embryos in the two experiments meant that the benchmarks of susceptibility and resistance were not the same in both experiments. Thus, direct comparison of the hybrid embryo results in each experiment was not valid. Rather, it was more meaningful to examine the similarities between the hybrid embryo responses and the CI or AW embryo responses within each experiment.

In both experiments, hybrid embryos exposed to AW sediment tended to respond similarly to CI embryos with relatively high average CVI values; however, this resemblance was not as strong in Experiment IV as it was in Experiment III. On Day 5, the average CVI value of the hybrid embryo/AW sediment treatment diverged substantially from the value of the CI embryo/AW sediment treatment in Experiment IV, while diverging only slightly in Experiment III. By Day 10, the difference between exposed CI and hybrid embryos in Experiment IV was much less pronounced.

The fact that the hybrids in both experiments responded relatively similarly to CI embryos supports the hypothesis that susceptibility to the teratogenic effects of the contaminated AW sediment is dominant over resistance in these embryos. In addition, the divergence in response between hybrid and CI embryos in Experiment IV as compared to Experiment III is consistent with the hypothesis that maternal effects also may be involved in moderating the expression of the susceptibility phenotype. It is difficult to draw any firm conclusions from these results, however, given the variability in embryo response observed within certain treatments in these experiments. Additional studies with larger sample sizes would be required to test the proposed hypotheses more thoroughly.

While the resistance trait appears to be affected by maternal factors, it does not appear to be sex-linked. If a trait is sex-linked, then reciprocal hybrid crosses should give very different results (Suzuki et al. 1986). For example, if it is assumed that CI fish carry an X-linked dominant susceptibility trait, then all of the progeny of the CI female x AW male cross should exhibit the dominant susceptible phenotype. This agrees with the results observed in Experiment III. Conversely, however, when AW females are crossed with CI males, all of the female progeny should exhibit the dominant susceptible phenotype while all of the male progeny should exhibit the recessive resistant

phenotype. This would result in an approximate 50:50 distribution of susceptible and resistant embryos (assuming a sex ratio of approximately 1:1), which was not observed in Experiment IV. While there were a few more susceptible hybrid embryos in that experiment than in the previous one, the proportion never approached fifty percent. For this reason, it is hypothesized that the resistance trait is likely to be autosomal rather than sex-linked.

Comparisons of the responses of hybrid and purebred embryos to contaminated sediment are highly dependent on the toxicant concentration in the sediment. For example, the differences in response between hybrid and CI embryos observed in Experiment IV might have been even more obvious if a slightly lower dose of AW sediment had been used. On the other hand, those differences might have disappeared if a higher dose of contaminated sediment had been used. This type of effect was observed in Experiment I, when AW embryos exposed to the medium and high doses of AW sediment exhibited significantly more mortality than embryos exposed to less contaminated sediment. Thus, in the medium and high dose treatments, the differences between AW and CI embryos were not as large as they were with the lower doses (although they still were very significant). For that reason, the low dose of contaminated AW sediment was used in the remaining experiments of this study, since it seemed to discriminate best between resistant AW and susceptible CI

embryos. If a more detailed analysis of the differences between hybrid and CI embryos is required, then it might be appropriate to choose a lower contaminant dose to allow for better discrimination between these two groups.

Most studies of xenobiotic susceptibility in experimental animals suggest that it is under polygenic control (Kleeberger and Levitt 1991). There are a few exceptions, however, in which Mendelian inheritance has been demonstrated. For example, Taylor et al. (1973) showed that resistance to cadmium-induced testicular necrosis in inbred mice is controlled by a single autosomal recessive gene. In one of the very few studies on the inheritance of toxicity resistance in fish, Yarbrough et al. (1986) demonstrated that cyclodiene insecticide resistance in mosquitofish is inherited as a single, autosomal, intermediate gene.

The results of the present study do not allow any speculation as to whether the contaminant resistance trait in the mummichog is controlled by one or several genes. Time constraints and difficulties in maintaining AW fish in the laboratory for extended periods precluded the production of the F_2 and backcross hybrids that would be needed to determine the mode of inheritance of the resistance trait. In addition, more information on the actual mechanism of resistance would be required to identify the specific phenotypes that best reflect the underlying resistant or susceptible genotypes in the embryos under study. While

mortality and cardiovascular malformation are useful end points of toxicity, they are complex characteristics that are likely to be influenced by factors other than genotype.

Only one other study has examined the inheritance of toxicity resistance in mummichog embryos. In that study, Toppin et al. (1987) performed reciprocal crosses with females and males from two populations that had shown significant differences in embryo resistance to methylmercury. Their results indicated that the male plays no role in the determination of embryo resistance. They attributed this finding to the fact that paternal gene products are just beginning to be synthesized during the early period of embryo development that is critical in the formation of abnormalities (Wilde and Crawford 1967; Weis and Weis 1989a). The resistance demonstrated by embryos from one of the populations was thought to be due to a faster initial development rate and decreased chorionic permeability; two factors that are determined by the female. However, Weis and Weis (1989a) noted that since embryos from this population were not similarly tolerant of other toxicants, chorionic impermeability could not be the major resistance mechanism, unless the chorion is selectively more impermeable to methylmercury.

In the present study, males were shown to play a role in determining resistance in their offspring. For example, embryos obtained from crossing an AW female with an AW male

were significantly more resistant to the toxic effects of contaminated sediment than were embryos obtained from an AW female by CI male cross. This suggests that, in this case, a sufficient amount of the paternal gene products required for resistance are synthesized fairly early in development.

The maternal effect that appears to moderate the expression of the susceptibility phenotype in the hybrid embryos might be attributable to differences in chorionic permeability. For example, AW females may produce eggs that are more impermeable to the toxicants in AW sediment than are CI eggs. In contrast, however, a faster developmental rate is not likely to explain the maternal effect, because no major differences in rates of development were noted among the various groups of embryos examined in this study.

Ecological Implications

Resistance to the acute toxicity of a contaminated environment, as demonstrated in mummichog at the AW site, does not come without a cost. Mulvey and Diamond (1991) suggested that adaptation to the environmental conditions at a polluted site is likely to involve reduced fitness in the initial, nonpolluted environment. Luoma (1977) cited work done on toxicity resistance in grasses and insects, which showed that resistance reduces the overall fitness of a population. When the selective pressure imposed by the

toxicant is removed, resistant populations usually revert to being dominated by susceptible genotypes.

In support of this theory, Weis and Weis (1989a) noted that methylmercury tolerance in mummichog embryos from a polluted site apparently comes at the cost of decreased tolerance of salinity and inorganic mercury in the embryos, and slower growth and weakness as adults. Some of the signs of stress seen in the adults include early reproduction, reduced growth and regeneration rates, reduced longevity and less feeding. The authors suggested that the weakened condition of the adults could merely reflect the stress of living in a contaminated environment, or it could be due to a lower level of genetic fitness that normally prevents resistant genotypes from dominating populations in uncontaminated environments.

There are some indications that mummichog at the AW site also may be paying a price for their increased toxicity resistance. These fish are much more difficult to maintain in the laboratory than are control fish, especially at elevated temperatures. It appears that these difficulties are due in part to an increased susceptibility to parasitic infestation in the AW fish, although controlled studies would be necessary to test this hypothesis. In addition, as noted in the Introduction, the AW mummichog exhibit an extremely high prevalence of liver cancer (Vogelbein et al. 1990). It is possible that susceptibility to

hepatocarcinogenesis may be one of the costs of acute toxicity resistance. An interesting theory that might be relevant in this case suggests that cancer might not be a separate phenomenon, but rather a deviant of the adaptive response of an organism to environmental xenobiotics (Farber and Rubin 1991).

Furthermore, the fact that toxicity resistance apparently is a relative rare trait in mummichog from uncontaminated environments could imply that there are some costs to being resistant. The tolerant genotype(s) may have a reduced level of fitness, which might explain why resistant CI embryos were not observed in any of the experiments in this study. It is unlikely that the relative lack of resistant genotypes is unique to the CI site, as similar results were observed in the preliminary experiments using embryos from the King Creek (KC) reference site.

The development of toxicity resistance in a fish population can have long-term detrimental effects in addition to short-term costs. Tolerance acquisition usually is associated with a narrow range of phenotypes and thus a reduction in the genetic variability of the exposed population (Mulvey and Diamond 1991). Without this variability, it is more difficult for the population to adapt to additional changes in the environment, such as those relating to the weather, climate patterns or human intervention (Mulvey and Diamond 1991; Longwell et al.

1992). Thus, the long-term evolutionary potential of a population can be reduced greatly if it is forced to acquire toxicity resistance to ensure its immediate survival.

CONCLUSIONS

The results of this study supported the hypothesis that mummichog embryos from a chemically contaminated site in the Elizabeth River, Virginia, are more resistant to the acute toxicity of contaminated sediment than are embryos from an uncontaminated reference site. This resistance was apparent when either mortality or cardiovascular malformation was used as a criterion for toxic effects. Nonresistant embryos exhibited a suite of cardiovascular terata, similar to that described in other studies of the developmental effects of various toxicants.

The observed resistance was shown to be due to genetic adaptation rather than physiological acclimation. It appears to be inherited as an autosomal recessive trait that may be modified by maternal effects.

FURTHER RESEARCH

The results of this study suggested several avenues for further research on such topics as exposure methodology, the genetics of resistance in mummichog populations, and possible resistance mechanisms. Some suggestions for potentially interesting and useful projects are described below.

1) Refinement of the flow-through exposure system The system developed by Sved (1991) and modified for use in the first preliminary experiment of the present study could be of value in future exposure studies if a few technical difficulties (described earlier in the Methods and Results sections) could be overcome. Given the prevalence of chemically contaminated sediments in aquatic environments, it would be a great advantage to have a relatively simple and reliable system for exposing test organisms to sediments in an environmentally realistic manner. For example, such a system would be ideal for conducting exposure experiments with larvae and adult fish as described below.

2) Determination of the degree of toxicity resistance in larvae and adult mummichog from the AW site While this study demonstrated that AW mummichog embryos are resistant to the acute toxicity of the contaminated AW sediment, it did not address the issue of resistance in later life stages of this fish. Weis et al. (1987) found that methylmercury tolerance in mummichog from a polluted environment was a stage-specific phenomenon. Embryos from this site were resistant to the toxicity of methylmercury, but larvae were only slightly resistant and adults were actually less resistant than were fish from a reference site. In contrast, preliminary work at this institution (Vogelbein and Van Veld, unpublished results; Williams, unpublished results) suggested that AW larvae and subadult fish retain a level of resistance similar to that seen in embryos. However, controlled experiments comparing the responses of AW larvae and adults to contaminated sediment with those of CI fish would be required to test this hypothesis.

3) Investigations into the genetics of the observed resistance The final two experiments of the present study using F1 hybrid embryos from reciprocal crosses of AW and CI mummichog provided some insight into the inheritance of the resistance trait in these fish. However, these experiments need to be repeated with larger sample sizes before any firm conclusions can be drawn from the observed results. In

addition, studies examining toxicity resistance in F2 hybrids and backcross progeny (F1 hybrids x AW or CI purebreds) are required in order to investigate more fully the type of genetic control involved in the inheritance of this trait. For this purpose, four groups of embryos have been maintained in the laboratory for the past year: AW purebreds, CI purebreds, AW female x CI male hybrids, and CI female x AW male hybrids. If these fish can be kept healthy and brought to breeding condition, they could provide the gametes necessary to perform the above-mentioned experiments.

Population level studies also could be of interest in examining the genetic basis of the observed resistance. If selection for resistant fish is occurring in a population in a polluted environment (such as AW), it should be detectable by a shift in the genetic structure of the population (Luoma 1977; Mulvey and Diamond 1991). Allozyme electrophoresis and/or restriction fragment length polymorphism (RFLP) analysis of mitochondrial DNA could be used to test the hypothesis that a genetic shift has occurred in the AW population as compared to other non-resistant mummichog populations in the area.

The levels of toxicity resistance in several mummichog populations would have to be determined to decide which ones should be included in the genetic structure study. The results of such a survey also would be of interest in

determining if the resistance observed in AW fish is a relatively unique or widespread phenomenon. In two preliminary experiments (Williams, unpublished results), mummichog embryos from a moderately contaminated site located directly across the Elizabeth River from the AW site demonstrated a degree of resistance to contaminated AW sediment. These results could imply that the level of contamination at the site was sufficient for the development of resistance in the local mummichog population or that there is genetic flow between this site and the AW site. Before any conclusions can be drawn, however, the data need to analyzed statistically and the experiment should be repeated with a larger sample size.

4) Investigations into possible mechanisms for the observed resistance The present study was not designed to examine the mechanism(s) of toxicity resistance in the mummichog. However, now that it has been shown that AW mummichog embryos have a genetic adaptation for resistance to contaminated sediment, there are several approaches that can be used to provide insight into possible mechanisms for this resistance.

One approach would be to test the resistance of AW embryos to various chemicals to determine the specificity of the observed tolerance. The creosote treatment used in the first experiment of the present study provided some

preliminary information in this area, but as stated in the Discussion, it needs to be repeated with larger amounts of creosote and better chemical analysis. In addition, a high molecular weight fraction of creosote could be tested, as it seems to have a PAH composition similar to that of AW sediment. Other chemicals that could be tested include single PAH, pesticides, and various metals.

A second approach would be to analyze the toxicant levels in various organs of exposed AW fish as well as in eggs from these fish, again using total PAH as an index of contamination. This type of study would provide information as to whether or not mummichog eggs are exposed to contaminants while developing within the female fish. In addition, AW and CI embryos could be analyzed for total PAH after exposure to AW sediment, to determine the degree to which each type of embryo accumulates toxicants and possibly the location of that accumulation (i.e., on the chorion or within the embryo). Similarly, the responses of dechorionated embryos to contaminated sediment could be compared to those of chorionated embryos to examine the role of the chorion in toxicity resistance.

Finally, the critical developmental stage at which toxicant exposure causes cardiovascular malformation could be determined by a series of exposure experiments using embryos of different ages. The results of such a study would provide information as to when the resistance

mechanism must be active in order to prevent developmental abnormalities. In a related experiment, immunohistochemistry could be performed on embryos of various ages to determine when (if ever) some of the known xenobiotic metabolizing enzymes are present in the developing embryo.

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VITA

CYNTHIA A. H. WILLIAMS

Born in Norristown, Pennsylvania, 2 June 1962. Graduated as valedictorian from Wayzata Senior High School in 1980. Attended Texas Christian University, majoring in pre-medical studies, from 1980-1982. Earned B.S. (*summa cum laude*, Phi Beta Kappa) in Biology from the College of Biological Sciences of the University of Minnesota in 1984. Entered master of arts program in the College of William and Mary, School of Marine Science in 1990.